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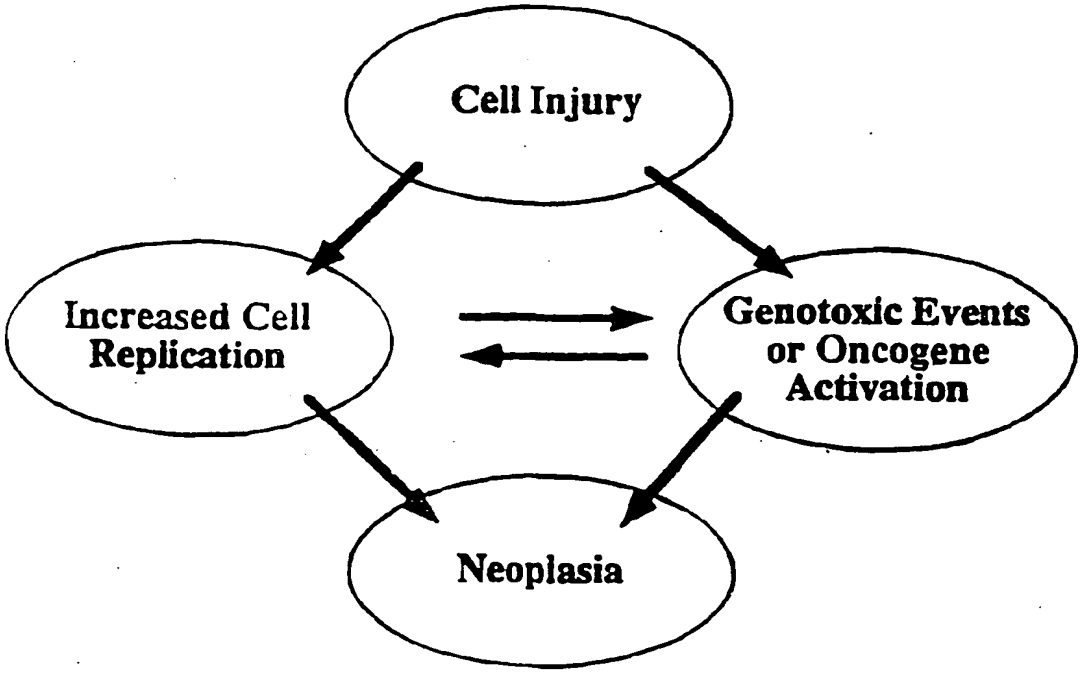

(57) Abstract

In vivo and (in vitro) assays are disclosed for measuring cyclin dependent kinase concentrations in cells or tissues, methods are provided for their use, including the use of such assays to evaluate carcinogenicity of a test compound, potential antineoplastic agents, and effectiveness of regions for increasing cell growth.

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PRODUCTS FOR MEASURING CELL GROWTH PROPENSITY AND METHODS FOR THEIR USE

This is a continuation-in-part application of U.S. Patent Application Serial No. 08/075,744, filed June 11, 1993, which in turn is a continuation-in-part application of U.S. Patent Application Serial No. 08/007,636, filed January 21, 1993.

FIELD OF THE INVENTION

This invention relates to *in vivo* and *in vitro* assays for measuring cell growth propensity and numerous applications therefor, including but not limited to the detection and quantification of substances, which are carcinogenic, even if the substances exhibit negative results in genotoxicity or mutagenicity tests.

BACKGROUND OF THE INVENTION

Introduction

Cell proliferation is the most fundamental phenotypic property of cancer. The stimulus for cellular proliferation is central not only at the late steps in carcinogenesis, the cancer, but also at the earliest known step, initiation (1,2) and Figure 1. In fact, cell proliferation exerts an influence in the initiation of carcinogenesis in that cells in the S phase are more sensitive toward many initiators than at other times in the cell cycle (3). A myriad of short-term tests exist for the assessment of the carcinogenic potential of

chemicals. These tests detect only carcinogens that interact with nucleic acids, or induce DNA repair synthesis or mutations in bacterial or mammalian cells (4-8).

As testing of the genotoxicity and carcinogenicity of chemicals has become routine, a growing number of compounds have been found to induce tumors in chronic bioassays while exhibiting negative results in genotoxicity tests (9). Significant examples of these classes of compounds include the dioxins, chlorinated biphenyls and peroxisome proliferators. These chemicals are often active as tumor promoters in two-stage experiments and exhibit biological activities as hormones (ethinylestradiol), peroxisome proliferators (pirnixic acid) or enzyme inducers (phenobarbital) (10).

At the present time only the initiation-promotion assay is employed routinely. In this assay the test compounds are examined for their ability to promote hepatic tumors or foci formation after initiation with a known genotoxic agent (11,12). As currently formatted, this assay utilizes animals, requires several months to perform, and produces histological endpoints that are difficult to quantify and do not lend to rigorous dose-response calculations for the purposes of risk assessment (13).

Stimulation of DNA synthesis has been proposed as an assay for short-term assessment of nongenotoxic carcinogens and tumor promoters *in vivo* (14,15). This methodology has potential for application to routine testing. So far, only one result has been detected that is inconsistent with carcinogenicity bioassay data. The different carcinogenicity of di(2-ethylhexyl)adipate (negative in rats) and di(2-ethylhexyl)phthalate

(positive) was not detectable by DNA stimulation index using ^{3}H-thymidine. Both plasticizers were positive in this short-term system with doubling doses of 0.7 mmol/kg for di(2-ethylhexyl)adipate and 0.5 mmol/kg for di(2-ethylhexyl)phthalate. Other disadvantages of this system include the use of radioactivity and the high coefficient of variation in the endpoint.

Several *in vitro* models have been utilized for the assessment of nongenotoxic carcinogens. Chida et al. (16) modeled the activation of protein kinase C and specific phosphorylation of a 90,000 kDa membrane protein of promotable BALB/3T3 and C3H/10T1/2 cells by tumor promoters. Smith and Colburn also utilized protein kinase C and its substrates in tumor promoter-sensitive and tumor-resistant cells as a biochemical marker for the response of cells to tumor promoters (17). However, these systems were flawed by both false positive and false negative values. The false positive values may be due to the fact that the activation of protein kinases C represents a biochemical signal far upstream from the final proliferative signal. While the false negatives may result from the fact that protein kinase C represents only a single receptor-mediated response. At least four other receptor responses, which are independent of protein kinase C, are known for tumor promotion and activity of nongenotoxic carcinogens (e.g. dioxin receptor, peroxisome proliferator receptor, phenobarbital receptor and estrogen receptor) (14,18).

Protein tyrosine phosphorylation

Protein-tyrosine kinases (PTK) constitute a class of enzymes that catalyze the transfer of the μ-

phosphate of either ATP or GTP to specific tyrosine residues in certain protein substrates. Evidence suggests that these enzymes are important mediators of normal cellular signal transduction (19-21), with PTK being the intracellular effectors for many growth hormone receptors (22-24). PTK are also frequently the products of proto-oncogenes (25) and their aberrant expression has been associated with a variety of human cancers (26).

The cascade of protein tyrosine phosphorylation following the activation of protein tyrosine kinases appears to regulate the proliferative response (27,28). Specific, protein tyrosylphosphorylations are common to a wide variety of nongenotoxic carcinogens independent of associated receptors or known mechanism of action. The present invention demonstrates the xenobiotic alterations in protein tyrosine phosphorylation at a fundamental point in the control of cellular proliferation and on an assay protocol that characterizes the ability of a xenobiotic test chemical to initiate cellular proliferation.

Cyclin-dependent Kinases (CDK)

Recent experimental evidence suggests that the cell cycle of all eukaryotic cells is controlled at several checkpoints by different members of a novel class of protein kinase, the cyclin-dependent kinases (29, 31, 36, 46). The most well known of these kinases is the 34 kD product of the cdc2 gene in the fission yeast $p34^{cdc2}$; however, several putative cyclin-dependent kinases (CDK) have now been cloned or identified. Some of these clones resemble $p34^{cdc2}$.

At least nine CDKs have been described in the literature; these all have a common PSTAIR epitope. Therefore anti-PSTAIR would be expected to cross react with the entire complement of CDKs showing up in the 32 to 34 kD region. (Apparently some cyclins also cross react with the anti-PSTAIR antibody and this explains the banding at approximately 60 kD observed in some of the immunoblots with anti-PSTAIR.)

The antibody to the C-terminus region is more specific for $p34^{cdc2}$ kinase, since the C-terminus region is more variable than the highly conserved PSTAIR region. However, it is obviously not species-specific since it was generated against human cdc2 and it cross reacts with mouse, rat and dog $p34^{cdc2}$ kinase.

SUMMARY OF THE INVENTION

In one embodiment, the invention provides a method for determining growth propensity for a tissue sample or cell line, said method comprising measuring a parameter that is indicative of concentration, in said sample or cell line, of at least one cyclin dependent kinase, and correlating said growth propensity to said measurement. Kits for carrying out this method are also provided.

Another embodiment of the invention provides a diagnostic method for determining whether a tissue or cell sample has undergone transformation to a cancerous phenotype, said method comprising measuring a parameter indicative of concentration, in said tissue or cell sample, of at least one cyclin dependent kinase, and correlating said measurement to likelihood of transformation

Another embodiment of the invention provides a diagnostic method for determining a likelihood that a tissue or cell sample will undergo transformation to a cancerous phenotype, said method comprising measuring a parameter that is indicative of concentration, in said tissue or cell sample, of at least one cyclin dependent kinase, and correlating said likelihood to said measurement.

Another embodiment of the invention provides a method of measuring carcinogenicity of a test substance comprising contacting said test substance with cells or tissue capable of expressing cyclin dependent kinase and thereafter measuring a parameter indicative of concentration, in said cells or tissue, of at least one cyclin dependent kinase, and correlating said carcinogenicity with said measurement.

Another embodiment of the invention provides a method of measuring effectiveness of a putative antineoplastic agent comprising the steps of:

(A) providing a sample of transformed cells;

(B) contacting said transformed cells with said putative antineoplastic agent;

(C) measuring a parameter indicative of concentration, in said cells, of at least one cyclin dependent kinase; and

(D) determining whether, or to what extent, said measurement indicates a decrease in cyclin dependent kinase following step (B).

Another embodiment of the invention provides a kit for measuring cyclin dependent kinase concentration in human or animal cell lysates, said kit including antibodies to an antigen whose concentration is

indicative of cyclin dependent kinase concentration in said lysates.

Another embodiment of the invention provides an immunohistochemistry kit for determining whether cells or tissues have undergone transformation to a cancerous phenotype or are likely to undergo such transformation, said kit comprising a slide for receiving a thin tissue slice containing said cells and further comprising an antibody to an antigen whose concentration is indicative of concentration of at least one cyclin dependent kinase in said cell or tissue sample.

Another embodiment of the invention provides a method for determining efficacy of a regimen for reducing or enhancing cell growth, said method comprising the steps of measuring a parameter indicative of concentration levels of at least one cyclin dependent kinase following treatment of those cells with said regimen and correlating cyclin dependent kinase concentration with said efficacy.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic of the multistage nature of carcinogenesis. Nongenotoxic carcinogens and tumor promoters affect, respectively, defects in terminal differentiation and selective clonal expansion of initiated cells.

Figure 2. A representation of the relationship between cell injury and neoplasia. The role of cell proliferation is characterized by the increased cell replication step.

Figure 3. The cell cycle. A cell can either be quiescent or continue to grow. The decision point is early in the G1 phase when a cell either passes START -

and then is committed to growing, finishing the rest of the cycle and dividing (G1, S, G2 and M) - or the cell enter the G_0 state in which it continues to metabolize but does not grow.

Figure 4. Immunoblot using anti-PSTAIR antibody. An anti-phosphotyrosine immunoprecipitate of the murine hepatic S-9 protein is separated using an 11% SDS-PAGE gel. The separated proteins are transferred to a blotting membrane and probed with the anti-PSTAIR antibody.

Figure 5. Scanning densitometry of anti-PSTAIR immunoblots for hepatic S-9 fraction of 2,3,7,8-tetrachlorodibenzo-p-dioxin treated female, C57BL/6J mice.

Figure 6. Bar graph depicting the quantification of the results of the scanning densitometry. The cyclin dependent kinase (CDK) quantified from the anti-PSTAIR immunoblot was at 32 kDa. The administration of a single dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin results in enhanced tyrosylphosphorylation of the CDK compared to control animals, which exhibit no tyrosylphosphorylation of CDK. Each group on the graph represents the single result of scanning an anti-PSTAIR immunoblot produced from the pooled hepatic S-9 of three animals. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 7. A typical BIAcore® sensorgram produced on immobilization of anti-cdc2 kinase C-terminus.

Figure 8. Anti-phosphotyrosine immunoblots of rat hepatic S-9 protein separated using 11% SDS-PAGE gels

for pirnixic acid-treated (lanes 1,2) and control (3,4) rats. Each lane represents a single rat.

Figure 9. Scanning densitometry of anti-phosphotyrosine immunoblots for pirnixic acid-treated rats [A and B] and paired vehicle controls [C and D, respectively]. Bolding of peaks indicates difference of greater than 40 percent between treatment and control.

Figure 10. Bar graph depicting the quantification of the results of the scanning densitometry. The phosphotyrosyl protein quantified from the anti-phosphotyrosine immunoblot was at 33 kDa. Results indicate that the administration of five, twice-daily doses of pirnixic acid (50 mg/kg each dose) produces enhanced tyrosylphosphorylation of p33 compared to control animals, which exhibit no tyrosylphosphorylation at 33 kDa. Each group on the graph represents the average of two rats. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 11. BIAcore® sensorgram displaying binding of pirnixic acid-treated S-9 protein and control S-9 protein over immobilized anti-cdc2 PSTAIR monoclonal antibody.

Figure 12. Summary bar graph depicting BIAcore® quantification of the interaction of tyrosylphosphorylated cyclin dependent kinases (CDK) with anti-CDK monoclonal antibodies (PSTAIR and C-terminus) from control and pirnixic acid-treated rats. Error bars represent standard deviations of n = 6 (anti-PSTAIR) and n = 8 (anti-C Terminus) control rats. RU (response units) value for pirnixic acid-treated rats represents the mean of 2 animals. The treatment of rats with 50 mg pirnixic acid/kg twice a day for 5 days results in

enhanced tyrosylphosphorylation of CDK ($p34^{cdc2}$ kinase) compared to control rats.

Figure 13. Anti-phosphotyrosine immunoblots of rat hepatic S-9 protein separated using 11% SDS-PAGE gels for diethylhexylphthalate-treated (lanes 1,2) and control (3,4) rats. Each lane represents a single rat.

Figure 14. Scanning densitometry of anti-phosphotyrosine immunoblots for diethylhexylphthalate-treated rats [A and B] and paired vehicle controls [C and D, respectively]. Bolding of peaks indicates difference of greater than 40 percent between treatment and control.

Figure 15. Bar graph depicting the quantification of the results of the scanning densitometry. The phosphotyrosyl protein quantified from the anti-phosphotyrosine immunoblot was at 34 kDa. Results indicate that the administration of five, twice-daily doses of diethylhexylphthalate (500 mg/kg each dose) produces enhanced tyrosylphosphorylation of the p34 compared to control animals, which exhibit no tyrosylphosphorylation at 34 kDa. Each group on the graph represents the average of two rats. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 16. Summary bar graph depicting BIAcore® quantification of the interaction of tyrosylphosphorylated cyclin dependent kinases (CDK) with anti-CDK monoclonal antibodies (PSTAIR and C-terminus) from control and diethylhexylphthalate-treated rats. Error bars represent standard deviations of n = 6 (anti-PSTAIR) and n = 8 (anti-C Terminus) control rats. RU value for diethylhexylphthalate-treated rats represents the mean of 2 animals. The treatment of rats with 500 mg diethylhexylphthalate/kg twice a day for 5 days produces

enhanced tyrosylphosphorylation of CDK ($p34^{cdc2}$ kinase) compared to control rats.

Figure 17. Anti-phosphotyrosine immunoblots of rat hepatic S-9 protein separated using 11% SDS-PAGE gels for diethylnitrosamine-treated (lanes 3,4) and control (lanes 1,2) rats.

Figure 18. Scanning densitometry of anti-phosphotyrosine immunoblots for diethylnitrosamine-treated rats [A and B] and paired vehicle controls [C and D, respectively]. Bolding of peaks indicates difference of greater than 40 percent between treatment and control.

Figure 19. Bar graph depicting the quantification of the results of the scanning densitometry. The phosphotyrosyl protein quantified from the anti-phosphotyrosine immunoblot was at 34 kDa. Results indicate that the administration of five, twice-daily doses of diethylnitrosamine (500 mg/kg each dose) produces no enhanced tyrosylphosphorylation of p34 compared to control animals. Each group on the graph represents the average of two rats. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 20. Summary bar graph depicting BIAcore® quantification of the interaction of tyrosylphosphorylated cyclin dependent kinases (CDK) with anti-CDK polyclonal antibodies (PSTAIR and C-terminus) from control and diethylnitrosamine-treated rats. Error bars represent standard deviations of n = 6 (anti-PSTAIR) and n = 8 (anti-C-terminus) control rats. RU value for diethylnitrosamine-treated rats represents the mean of 2 animals. Results indicate that the treatment of rats with 500 mg diethylnitrosamine/kg twice a day for 5 days

produces no enhanced tyrosylphosphorylation of CDK ($p34^{cdc2}$ kinase) compared to control rats.

Figure 21. Anti-phosphotyrosine immunoblot of dog hepatic S-9 protein separated using 11% SDS-PAGE gels for Aroclor®-treated dogs. Lanes 1, 2, 3, 4 and 5 are control, 0.6, 0.8, 4 - 8, and 5 - 10 mg Aroclor®/kg-day, respectively.

Figure 22. Scanning densitometry of anti-phosphotyrosine immunoblots at 34 kDa for Aroclor®-treated dogs. From top to bottom the figures represent 0.6, 0.8, 4 - 8, and 5 - 10 mg Aroclor®/kg-day, respectively.

Figure 23. Bar graph depicting the quantification of the scanning densitometry of the putative cyclin dependent kinase (p34) from the anti-phosphotyrosine immunoblot. The daily administration of Aroclor® for a period of 11.5 weeks results in enhanced tyrosylphosphorylation of the p34 at all doses compared to the control dog. Each bar on the graph represents the result of scanning an immunoblot produced from the hepatic S-9 of a single dog. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 24. Anti-phosphotyrosine immunoblots of 3T3 cell lysate protein separated using 11% SDS-PAGE gels for 3T3 cells exposed to 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (lane 3B) or DMSO vehicle (lane 1B) for 24 h in 0.5% serum supplemented media.

Figure 25. Scanning densitometry of anti-phosphotyrosine immunoblots for 3T3 cells treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or DMSO vehicle (Control) for 24 h in 0.5% serum media. Bolded peaks indicate p34 and p33 tyrosylphosphoproteins.

Figure 26. Bar graph depicting the quantification of the scanning densitometry of the putative cyclin dependent kinases (p34/p33) from the anti-phosphotyrosine immunoblot. Exposure of 3T3 cells to 10 nM 2,3,7,8-tetracholordibenzo-p-dioxin for 24 h results in an increase in tyrosylphosphorylation of p34 and p33 of 67 and 32%, respectively, compared to the vehicle control. Each bar on the graph represents the result of scanning an immunoblot produced from the pooled whole cell lysates of four plates per treatment. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 27. Anti-phosphotyrosine immunoblots of 3T3 cell lysate protein separated using 11% SDS-PAGE gels for 3T3 cells exposed to 160 nM 12-O-tetra-decanoylphorbol-13-acetate (TPA; lane 4B) or DMSO vehicle (Control; lane 1B) for 24 h in 0.5% serum supplemented media.

Figure 28. Scanning densitometry of anti-phosphotyrosine immunoblots for 3T3 cells treated with 160 nM 12-O-tetra-decanoylphorbol-13-acetate (TPA) or DMSO vehicle for 24 h in 0.5% serum media. Bolded peaks indicate p34 and p33 tyrosylphosphoproteins.

Figure 29. Bar graph depicting the quantification of the scanning densitometry of the putative cyclin dependent kinases (p34/p33) from the anti-phosphotyrosine immunoblot. Exposure of 3T3 cells to 160 nM 12-O-tetra-decanoylphorbol-13-acetate (TPA) for 24 h results in an increase in tyrosylphosphorylation of p34 and p33 of 54 and 95%, respectively, compared to the vehicle control. Each bar on the graph represents the result of scanning an immunoblot produced from the pooled whole cell lysates of four plates per treatment. Error

bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 30. Anti-phosphotyrosine immunoblots of BNL CL.2 cell lysate protein separated using 11% SDS-PAGE gels for BNL CL.2 cells exposed to 0.1, 1, 10, or 100 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; lanes 3,4,5 and 6, respectively) or DMSO vehicle (lane 1) for 24 h in 0.5% serum supplemented media. Lane 2 is the 20% serum-supplemented control.

Figure 31. Scanning densitometry of anti-phosphotyrosine immunoblots in the 35 to 30 kDa molecular weight range for BNL CL.2 cells treated with 0.1, 1, 10 or 100 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or DMSO vehicle (Control) for 24 h in 0.5% serum supplemented media. p34 and p33 tyrosylphosphoproteins are indicated for the respective treatments. Top row (left to right) 0.5 % and 20% serum supplementation; Middle row 0.1 and 1 nM TCDD; Bottom row 10 and 100 nM TCDD.

Figure 32. Bar graphs depicting the quantification of the scanning densitometry of the putative cyclin dependent kinases (p34-top/p33-bottom) from the anti-phosphotyrosine immunoblot. Exposure of BNL CL2 cells to 0.1, 1, 10 or 100 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for 24 h results in a similar increase in tyrosylphosphorylation of p34, averaging 180% of the vehicle control over all concentrations of TCDD. Twenty percent serum supplementation results in an increase of tyrosylphosphorylation of p34 of 229% of the vehicle control. Vehicle controls at 0.5% serum supplementation exhibit no tyrosylphosphorylation at p33, while TCDD exposure at the four concentrations enhances

tyrosylphosphorylation of this putative CDK to 0.9, 2.0, 2,0 and 1.9 density units, respectively. The increases in tyrosylphosphorylation of p33 by TCDD are 3.4 times the p33 tyrosine phosphorylation produced by 20% serum supplementation. Each bar on the graph represents the result of scanning an immunoblot produced from the pooled whole cell lysates of four plates per treatment. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 33. Anti-phosphotyrosine immunoblots of BNL CL.2 cell lysate protein separated using 11% SDS-PAGE gels for BNL CL.2 cells exposed to 1, 10, 100, or 1000 nM pirnixic acid (lanes 7,8,9 and 10, respectively) or DMSO vehicle (lane 1) for 24 h in 0.5% serum supplemented media. Lane 2 is the 20% serum-supplemented control.

Figure 34. Scanning densitometry of anti-phosphotyrosine immunoblots in the 35 to 30 kDa molecular weight range for BNL CL.2 cells treated with 1, 10, 100, or 1000 nM pirnixic acid or DMSO vehicle (Control) for 24 h in 0.5% serum media. p34 and p33 tyrosylphosphoproteins are indicated for the respective treatments. Top row (left to right) 0.5 % serum and 20% serum; Middle row 1 and 10 nM pirnixic acid; Bottom row 100 and 1000 nM pirnixic acid.

Figure 35. Bar graphs depicting the quantification of the scanning densitometry of the putative cyclin dependent kinases (p34-top/p33-bottom) from the anti-phosphotyrosine immunoblot. Exposure of BNL CL2 cells to pirnixic acid for 24 h results in increases in tyrosylphosphorylation of p34 relative to the vehicle control for the 1 and 10 nM concentrations, 96 and 58% increases, respectively. At 100 nM pirnixic acid, the tyrosylphosphorylation of p34 is similar to the

vehicle control, while at 1000 nM tyrosine phosphorylation of p34 is depressed 60% from the vehicle control. Twenty percent serum supplementation results in an increase of tyrosylphosphorylation of p34 of 229%, relative to the vehicle control. The 0.5% serum supplementation control exhibits no tyrosylphosphorylation at p33, while pirnixic acid exposure enhances tyrosylphosphorylation of this putative CDK to 2.0, 2.5 and 0.5 density units, respectively, at the 1, 10, and 100 nM concentrations. The increases in tyrosylphosphorylation of p33 by pirnixic acid at 1 and 10 nM are roughly 4 times the p33 tyrosine phosphorylation produced by 20% serum supplementation. Each bar on the graph represents the result of scanning an immunoblot produced from the pooled whole cell lysates of four plates per treatment. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 36. Bar graph depicting the microtiter methodology for quantification of tyrosylphosphorylation of tissue CDK. The capture antibody was anti-PSTAIR and the secondary antibody was anti-phosphotyrosine. Dosing of C57BL/6J female mice daily with 0, 0.25, 0.5, 1 or 2 ng TCDD/kg-day (A, B, C and D, respectively) results in enhanced tyrosylphosphorylation of hepatic CDK but not pulmonary or renal CDK. This identifies the target tissue for the cellular proliferative effects of TCDD as the liver. Maximal increase in tyrosylphosphorylation of hepatic CDK is observed at the 0.5 ng TCDD/kg-day dose regimen. The error bars represent the 95 percent confidence interval of the mean absorbance determined at 415 nm for each of the treatments (n=10 mice per treatment).

Figure 37. Bar graph depicting the microtiter methodology for quantification of tyrosylphosphorylation of tissue $p34^{cdc2}$ kinase. The capture antibody was anti-C-terminus and the secondary antibody was anti-phosphotyrosine. Dosing of C57BL/6J female mice daily with 0, 0.25, 0.5, 1 or 2 ng TCDD/kg-day (A, B, C and D, respectively) results in enhanced tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase but not pulmonary or renal $p34^{cdc3}$ kinase. This identifies the target tissue for the cellular proliverative effects of TCDD as the liver. Maximal increase in tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase is observed at the 0.5 ng TCDD/kg-day dose regimen. The error bars represent the 95 percent confidence interval of the mean absorbance determined at 415 nm for each of the treatments (n=10 mice per treatment).

Figure 38. The anti-cdc2 C-terminus immunoblot of rat hepatic S9 proteins separated using 10 to 11% SDS-PAGE gels for control (lanes 1 and 3) and WY 14,643-treated rats (lanes 2 and 4). A single intensely-stained band was visible in the CDK region (32 to 35 kDa) in hepatic S9 samples obtained from rats three days after receiving a single does of 50 mg WY14,643/kg. This band is barely visible in hepatic S9 from control rats.

Figure 39. Bar graph depicting the microtiter methodology for quantification of CDK expression in rat liver S9. The treated rats receive a single does of 50 mg pirnixic acid/kg and are killed 1, 2 or 3 days later; control rats are dosed with the vehicle alone. The mean absorbance developed at 415 nm over 10 min is presented on the y-axis. Error bars represent standard deviations of n = 4 (1 day) and n = 5 (2 and 3 day) rats per treatment. The extent of CDK expression the livers

of young, male rats receiving a single does of 50 mg/kg of WY 14,643 increases steadily during the 3-day postdosing observation period. CDK expression in control animals remains constant over the same 3-day period.

Figure 40. Anti-cdc2 C-terminus immunoblot of BNL CL.2 cell lysate protein separated using 10 to 11% SDS-Page gels for BNL CL.2 cells exposed to 0.1, 1, or 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioin (TCDD; lanes 8, 9, and 10, respectively) or DMSO vehicle (lane 6) for 48 h in 0.5% serum supplemented media. Lane 7 is the 20% serum-supplemented control. TCDD exposure results in increased expression of CDK relative to the DMSO control.

DETAILED DESCRIPTION OF CERTAIN PREFERRED EMBODIMENTS

In accordance with the present invention, there is provided novel methods, and kits for performing the methods, for measuring parameters indicative of the concentration of at least one cyclin dependent kinase in human or animal tissues, cell lines, cell lysates, tissue homogenates and the like. Applicants have discovered a relationship between cyclin dependent kinase concentration and cell growth (or propensity therefor). Cell proliferation being the most fundamental phenotypic property of cancer, the present invention has broad application to, *inter alia*, determining whether cells or tissue have transformed to a cancerous phenotype, determining the likelihood of such transformation later occurring, detecting and quantifying carcinogenicity of test substances (even substances which are nongenotoxic and/or nonmutagenic), testing putative antineoplastic agents, etc.

The invention also has broader applications in determining cell growth in general, and in evaluating the

effectiveness of regimens designed to increase or decrease cell growth. Without intending to be bound by theory, it is believed that the concentration of cyclin dependent kinase is indicative of the proportion of cells which are out of the G. phase of their cell cycle. Thus, measuring cyclin dependent kinase concentration (or a related parameter) provides a very early indication of increased cell growth (or a propensity therefor) significantly sooner than cell growth or cell transformation can be observed utilizing most other techniques.

In accordance with the invention, cyclin dependent kinase may either be measured directly or, alternatively, by measuring other parameters which are indicative of cyclin dependent kinase concentration. These other parameters may be parameters which vary with cyclin dependent kinase concentration, or even parameters which vary inversely with cyclin dependent kinase. For example, in some embodiments of the invention, parameters are measured which are related to either the formation or later metabolic fate of cyclin dependent kinase. For example, mRNA for cyclin dependent kinase could be measured, as could proteases involved in the degradation of cyclin dependent kinase. In one embodiment, tyrosylphosphorylation of cyclin dependent kinase is measured. The foregoing measurements are preferably performed by ELISA or immunohistochemical techniques utilizing antibodies to at least one cyclin dependent kinase, or to other antigens the concentration of which is indicative of cyclin dependent kinase concentration (e.g., some of the related parameters discussed above).

After measurements are taken, it is preferred but not required that measurements be compared to a

control which may be either a historical or concurrent control, standard curve, archival materials, or the like. However, for a given purpose, a user's own prior experience with the measurement, and with its implications may be sufficient for subjective evaluation of the measurement by the user. In some embodiments of the invention, "before" and "after" measurements are taken to determine the effect of an intervening regimen or of exposure to stimulus. In other embodiments, abnormal measurement levels based on historical or archival data or standard curves are determined. Depending upon the sensitivity desired, for example, a positive indication could be set at one, two or three standard deviations above the mean of a normal control. Those of skill in the art will recognize a wide range of uses for the present methods and kits, only a representative sample of which are discussed below.

For purposes of diagnostic evaluation of tissues, samples suspected of having undergone transformation to cancerous phenotype, e.g., breast, prostate, colon, lung, stomach or pancreas tissues, or lymphocytes, etc., may be subjected to the methods of the present invention wherein abnormal measurements of parameters indicative of cyclin dependent kinase concentration will represent a positive signal for transformation to cancerous phenotype or likelihood of transformation. The ability to determine likelihood of transformation is of particular value in biopsy, especially when a patient is to undergo surgery for removal of a tumor. The present invention provides an improved method of determining how radical such surgery should be, and how much tissue should be removed.

Other applications include the diagnostic evaluation of potential agents to induce cancer phenotype in any cell or tissues. Comparative testing could be done, for example, utilizing fish or other animals from waters polluted with certain pollutants (the same animals from cleaner water could be used as controls).

The invention also has research applications to laboratory animals, and to providing model *in vitro* systems for the potency of carcinogenic agents or potential antineoplastic agents.

It is also possible, for example, to test the potency of potential inhibitors of various biological responses. A cell's response to a mitogen can be measured in accordance with the present invention, and the response to a combination of mitogen and a test inhibitor (at increasing concentrations) can also be tested by the present invention. The decrease in proliferation induced by the mitogen at increasing concentrations of inhibitor can be shown by measuring cyclin dependent kinase concentration in accordance with the invention, thereby providing a test of the effectiveness of the inhibitor.

The test of the present invention could also be used to establish "no effect" thresholds for toxic effects of various compounds. The test of the invention can be utilized, for example, to determine a threshold concentration below which the test compound will not interfere, for example, with the function of liver cells tested in accordance with the invention.

The present invention is able to provide statistically significant results after a very short period of time of cell incubation with a test compound. In preferred embodiments, *in vivo* tests involved

administering a test compound to a animal and allowing about 24 hours before sampling tissue. In *in vitro* tests, 24-48 hours of cell exposure to a test compound is preferred.

In certain embodiments of an *in vitro* test for carcinogenicity of a test compound, cells are synchronized at operational G. by deprivation of growth factors. The test compound is administered to some dishes, serum to a positive control, and nothing to a negative control. After about 24-48 hours, cells are harvested and lysed, the lysate being subjected to measurement of a parameter indicative of cyclin dependent kinase concentration in accordance with the invention. In one embodiment of this in vitro test, the cells to be tested are lysed (they should be kept cold through the procedure). Preferably, they are kept on ice and their temperature does not exceed 2-4°C. Both sample and standards are then bound to the plate, after diluting with a sample dilution buffer, e.g. a sodium borate buffer at pH 10.5 (about 100mM). The protein concentration is preferably between 6 and 100 μg per ml of dilution buffer. In accordance with standard ELISA techniques, the above binding is preferably followed by blocking the remaining sites, adding the primary antibody (anti-PSTAIR by way of example only), adding the secondary antibody and color development (where the secondary antibody is detectable by color).

In a corresponding *in vivo* application, animal tissue is obtained about 24 hours after exposure to a test compound. The tissue is preferably slurried and then subjected to testing of a parameter indicative of cyclin dependent kinase concentration. One preferred embodiment proceeds like the *in vitro* test above.

Protein concentration for the *in vivo* test being preferably between 12.5 and 50 μg protein per ml of dilution buffer.

Preferred kits of the invention provide a lysate buffer for an *in vitro* test, or homogenization buffer for an *in vivo* test and a dilution buffer for both.

Immunohistochemical analysis of cells suspected of having transformed to a cancerous phenotype, or suspected of having increased susceptibility to transformation, may proceed in an analogous manner starting with a thin (e.g. 4-6 micron) sample immobilized on a slide that has preferably been microwaved for about 10 minutes. Positive and negative controls are preferably provided on the slide.

Naturally, it is preferred that the antibodies used are specific for the particular antigen being measured and that the antibody formulations are substantially free of contaminants and of other antibodies to avoid cross-reactivity. The antibodies may be, for example, anti- cyclin dependent kinase (when cyclin dependent kinase concentration is being measured directly). Preferred anti- cyclin dependent kinase includes but is not limited to anti-PSTAIR, and antibodies to cyclin dependent kinases having an apparent molecular weight between about 32 and 34 kD, especially 33 kD and 34 kD, when measured on polyacrylamide gel.

Possible substances that may be tested in accordance with the invention include peroxisome proliferators, estrogens, estrogen receptor, testosterone, testosterone receptor. The invention may also measure carcogenicity of compounds from the dioxin or PCB group.

As used herein, "cell samples" may include tissues that have the type of cells under discussion.

A method and assay to determine whether a test compound or sample is a nongenotoxic carcinogen, wherein the compound or sample to be tested is added to a cyclin dependent kinase (CDK) assay system is provided. The assay system can be inter alia a living organism, a cell culture or a cell lysate, as long as the assay system contains a cyclin dependent kinase (CDK). An increase in the tyrosylphosphorylation level of CDK (one indication of increased CDK concentration) indicates that the test compound is a nongenotoxic carcinogen, or that the test sample contains a nongenotoxic carcinogen.

This assay also detects nonmutagenic carcinogens and substances having a cell proliferation effect. The nongenotoxic carcinogens that can be identified through the assay include tumor promoters, chlorinated biphenyls, hormones, dioxins and peroxisome proliferators, among others. The assay system can be assembled in the form of a test kit for diagnostic and environmental testing.

The above assay could also be used to quantify the potency of a particular growth factor (peptide hormone). A peptide growth factor would be added to the assay system instead of a xenobiotic (foreign chemical) and otherwise the assay would proceed without modification.

The method and assay of the invention can also be used to determine the potential of a chemical as an antineoplastic agent by reversing the steps outlined above. Starting with a transformed cell or transformed cell lysate, a potential antineoplastic agent would be tested for the capacity of the chemical to put the cells

into the G_0 state. This capacity would be determined by quantifying the decrease in cyclin dependent kinase, e.g. by measuring tyrosylphosphorylation of the CDK. The only other modification necessary to convert the assay for nongenotoxic carcinogens to one for antineoplastic agents is to grow the neoplastic cells *in vitro* in a full serum complement (20% serum containing medium).

In Vivo Experiments

EXAMPLE 1

Enhanced tyrosylphosphorylation of $p34^{cdc2}$ kinase in an hepatic cytosol (S-9) preparation from C57BL/6J female mice 24 hours following administration of the nongenotoxic carcinogen 2,3,7,8-tetrachlordibenzo-p-dioxin.

Summary

$p34^{cdc2}$ is the serine/threonine kinase subunit of M-phase promoting factor (MPF) (29-31). The regulation of $p34^{cdc2}$ tyrosine phosphorylation status is considered the control mechanism for entry into G_1 from G_0, the START signal, and also from G_2 to M, the initiation of mitosis. It is demonstrated that a single dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin administered at 0.25, 0.5, 1, or 2 μg/kg to young, female mice increases the extent of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase compared to corn oil treated controls. These results indicate that the proliferative stimulus of the nongenotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin may be quantified as an increase in hepatic $p34^{cdc2}$ kinase tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase can serve to indicate the capacity of a dioxin-like chemical to function *in vivo* as a nongenotoxic carcinogen.

Materials and Methods

Chemicals: 2,3,7,8-tetrachlorodibenzao-p-dioxin (TCDD) is purchased from AccuStandard, Inc. (New Haven, CT).

Anti-phosphotyrosine monoclonal, anti-PSTAIR (CDK), and anti-p34^{cdc2} kinase C-terminus polyclonal antibodies are obtained from UBI (Lake Placid, NY). The acronym PSTAIR is the abbreviation for the amino acid sequence used as the antigen for developing the anti-PSTAIR antibody. The two antibodies (PSTAIR and anti-C-terminus) recognize two different epitopes. At least nine CDKs have been described in the literature; these all have a common PSTAIR epitope. Therefore anti-PSTAIR would be expected to cross react with the entire complement of CDKs showing up in the 32 to 34 kD region. (Apparently some cyclins also cross react with the anti-PSTAIR antibody and this explains the banding at approximately 60 kD observed in some of the immunoblots with anti-PSTAIR.)

The antibody to the C-terminus region is more specific for p34^{cdc2} kinase, since the C-terminus region is more variable than the highly conserved PSTAIR region. However, it is obviously not species-specific since it was generated against human cdc2 and it cross reacts with mouse, rat and dog p34^{cdc2} kinase.

One or the other antibody is used depending upon the specificity desired in the experiments.

Bicinchoninic acid is obtained from Pierce (Rockford, IL). Molecular weight standards are supplied through BioRad (Melville, NY). All other chemicals are

purchased from Sigma (St. Louis, MO) and are of the highest purity available.

Animals and dosing: Four to six-wk old, female C57BL/6J mice are obtained from Harton Sprague Dawley (Indianapolis, IN). The mice are fed Prolab RMH 1000 (Agway, Cortland, NY) and receive tap water *ad libitum*. All mice are housed three per cage and maintained on a photoperiod of 12 h. Mice are killed 24 h following an intraperitoneal injection of TCDD in corn oil at 0, 0.25, 0.5, 1, or 2 μg/kg. Three mice are treated at each dose and the volume of the injections ranges from 0.1 to 0.2 mL per mouse. All preparation procedures are performed on pooled hepatic samples of the three mice per dose.

Preparation and -80°C storage of hepatic S-9 fractions is performed exactly as previously described in the scientific literature (32). This procedure involves killing the mouse by cervical dislocation, removing the liver and homogenizing the liver in three volumes of 0.15 M KCl. This hepatic homogenate is centrifuged at 9,000 x g for 20 min at 4°C. The resulting supernatant fraction, termed the S-9, is decanted into 1.5 mL plastic, conical tubes, frozen in a dry ice/ethanol bath and stored at -80°C until immunoprecipitation of phosphotyrosyl proteins can be performed.

Immunoprecipitation of tyrosine phosphorylated hepatic S-9 proteins with anti-phosphotyrosine monoclonal antibody: The hepatic S-9 is solubilized in immunoprecipitation buffer containing 20 mM Tris HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.15 U/mL aprotinin, and 1 mM sodium vanidate, centrifuged at 13,000 x g for 15

min at 4°C. The solubilized hepatic S-9 proteins are then incubated with anti-phosphotyrosine monoclonal antibody (5 μg/mL) at 4°C for 4 h or overnight. After the incubation period, add 25 μL of protein A-Sepharose for each 5 μg of antibody. One h later the immune complexes are collected by centrifugation at 13,000 x g, washed twice with immunoprecipitation buffer, solubilized in SDS gel sample buffer and heated at 100°C for 5 min in preparation of SDS PAGE and immunoblotting.

Gel electrophoresis and immunoblotting: SDS PAGE is carried out as described in the scientific literature (33) using 11% polyacrylamide gels with the modification that hepatic S-9 (100 μg protein/well) are subjected to heat treatment (100°C) for 3 min. The immunoblotting assay is performed as described by Towbin et al. (34), however a Milliblot SDE electroblot apparatus (Millipore, Bedford, MA), is used to transfer proteins from polyacrylamide gels to an Immobilon® membrane filter (Millipore,Bedford, MA). Complete transfers are accomplished in 25-30 min at 500 mA and are assessed by tracking pre-stained molecular weight standards on the membrane filter.

Membrane filters are blocked by incubating in TBS (Tris buffered saline) containing 5% commercial nonfat dry milk (any commercial brand is suitable) for 30 min at room temperature. The membranes are then washed in TBST (TBS with 0.05% Tween 20) and incubated for 2 h with anti-human CDK (PSTAIR) antibody (2 - 5 μg/mL) in TBST or anti-mouse cdc2 kinase (C-terminus) polyclonal antibody in TBST. The antibody reaction is visualized by incubating the membranes for 2 h at room temperature with alkaline phosphatase-conjugated anti-mouse IgG diluted

1:1000 in TBST and developed for 15 min. Molecular weights are determined by adding molecular weight standards (Bio Rad, Melville, NY) to reference lanes and staining the membrane filters with amido black 10B. The resulting immunoblots are scanned into TIFF-formatted files (MacIntosh®; Apple Computers, Cupertino, CA) with a Microtech 600GS scanner (Torrance, CA) and quantified using Scan Analysis (BIOSOFT, Cambridge, UK). Summary scans are then printed and peak heights are measured directly from the figure. One density unit (U) is defined as one mm of the resulting peak height.

Protein determination: Bicinchoninic acid is used for the spectrophotometric determination of protein concentration (35). Mix 100 μL of sample (standard or unknown) with 2 mL of working reagent in a test tube. Color development occurs by incubation at 37oC for 30 min. Absorbance is read at 562 nm. Working reagent is made by adding 100 volumes of Reagent A with 2 volumes Reagent B. Reagent A: is made by combining 1.0 g bicinchoninic acid (Pierce Chemical, Rockford, IL); 2.0 g $Na_2CO_3*H_2O$; 0.16 g NaOH; and 0.95 g $NaHCO_3$ with water to 100 mL and adjust the pH to 11.25 with 50% NaOH. Reagent B consists of 4.9 g $CuSO_4*5H_2O$ to 100 mL in double distilled H_2O.

Results:

The anti-phosphotyrosine immunoprecipitate of the murine hepatic S-9 is run on an 11% polyacrylamide gel as described above and immunoblotting is performed with the anti-PSTAIR monoclonal antibody. The resulting anti-PSTAIR immunoblot is depicted in Figure 4. Density scans of the immunoblot are presented in Figure 5 and the

quantification of these bands is presented in Figure 6. The bands in Figure 4 at 34 and 32 kDa immunoreactive with anti-PSTAIR have been identified as cyclin dependent kinases and at this time it is not known if they represent isoforms of a single $pp34^{cdc2}$ kinase or whether they are two separate cyclin dependent kinases (36). The large anti-PSTAIR immunoreactive band at approximately 60 kDa has been identified as a cyclin protein (37, 38).

The results demonstrate that the tyrosylphosphorylated CDK ($pp34^{cdc2}$) does not exist in measurable quantities in the hepatic S-9 of corn oil treated control mice. However, dosing of mice with TCDD enhanced the tyrosylphosphorylation of a p34 and p32 to a maximum at 0.5 μg TCDD/kg. At higher doses of TCDD the tyrosylphosphorylation of the kinase(s) becomes attenuated, perhaps due to overt toxicity of TCDD to the mice at these higher doses.

EXAMPLE 2

Enhanced tyrosylphosphorylation of $p34^{cdc2}$ kinase in an hepatic cytosol preparation (S-9) from young male rats 24 hours following administration of the nongenotoxic carcinogen pirnixic acid.

Summary

$p34^{cdc2}$ is the serine/threonine kinase subunit of M-phase promoting factor (MPF) (29-31). The regulation of $p34^{cdc2}$ tyrosine phosphorylation status is considered the control mechanism for entry into G_1 from G_0, the START signal, and also from G_2 to M, the initiation of mitosis. It is demonstrated that twice daily doses of 50 mg pirnixic acid/kg of body weight for 5 days to young male rats increases the extent of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase compared to corn oil treated

controls. These results indicate that the proliferative stimulus of the nongenotoxic carcinogen pirnixic acid may be quantified as an increase in hepatic $p34^{cdc2}$ kinase tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase can serve to indicate the capacity of chemicals that are termed peroxisome proliferators to function *in vivo* as a nongenotoxic carcinogen.

Materials and Methods

Chemicals: Pirnixic acid (CAS 50892-23-4 [4-chloro-6-(2,3-xylidino)-2-pyrimidiylthio] acetic acid) is purchased from ChemSyn Science Labs (Lenexa, KY). Anti-phosphotyrosine monoclonal, anti-PSTAIR (CDK), and anti-$p34^{cdc2}$ kinase C-terminus polyclonal antibodies are obtained from UBI (Lake Placid, NY). Bicinchoninic acid is obtained from Pierce (Rockford, IL). Molecular weight standards are supplied through BioRad (Melville, NY). Sensor Chips CM5, Surfactant P20, and amine coupling kit (EDC, NHS, and ethanolamine hydrochloride) were purchased from Pharmacia Biosensor AB. All other chemicals are purchased from Sigma (St. Louis, MO) and are of the highest purity available.

Animals and dosing: Eight-wk old male Sprague-Dawley rats are purchased from Charles River Laboratory (Charles River, MA) and housed four to a cage in polycarbonate cages (24 x 34 x 20 cm). Bedding consists of hardwood chips. Rats are allowed free access to tap water and fed Agway RMH 3000 (Cortland, NY) *ad libitum*. Photoperiod is maintained at 12 h of light and 12 h of darkness.

After a wk of acclimation to new surroundings, treatments are begun. The treatment consists of twice daily doses of the test compound administered by oral gavage. The pirnixic acid is dissolved in corn oil. Sham-treated animals are given an equal volume of plain corn oil. Doses are adjusted daily on the basis of weight. The volume of corn oil is generally on the order of 2 mL/ rat throughout the treatment period. The second dose is given between the h of 13:00-16:00, approximately 6 h after the first dose given between the h of 7:00 - 10:00. The pirnixic acid is administered for 5 days at a dose of 50 mg/kg twice a day.

On the day of sacrifice the rats are anesthetized with ethyl ether and decapitated. Livers are removed, weighed and homogenized using a Potter-Elvehjem® tissue grinder with 3 mL of ice-cold 0.15 M KCl per g of wet weight of liver. This material is pooled for each rat and spun in a high speed centrifuge (Beckman J2-MI, Beckman Instruments, Fullerton, CA) for 10 min at 9000 x g at 4°C. The supernatant liquid is decanted, distributed as aliquot and frozen at -90°C.

Gel electrophoresis and immunoblotting with anti-phosphotyrosine: These procedures are carried out essentially as described in Example 1 except that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Real-time quantification of total tyrosylphosphorylated $p34^{cdc2}$ kinase: Surface plasmon

resonance (SPR) is used for the real time quantification of $p34^{cdc2}$ kinase that exists in the tyrosylphosphorylated form. SPR is sensitive to changes in the optical properties of a medium close to a metal surface (39). SPR is suitable for macromolecular interaction studies at solid/liquid interfaces with the use of a carboxymethylated dextran hydrogel placed upon a thin layer of gold (39,40).

The detection system of a SPR monitor consists of a light source emanating both monochromatic and plane-polarized light, a glass prism, a thin metal film in contact with the base of the prism, and a photodetector. An evanescent field forms from the prism into the metal film when obliquely incident light on the base of the prism will exhibit total internal reflection for angles greater than the critical angle. This evanescent field can couple to an electromagnetic surface wave, a surface plasmon, at the metal/liquid interface. Coupling is achieved at a specific angle of incidence, the SPR angle (39).

The SPR angle is highly sensitive to changes in the reactive index of a thin layer adjacent to the metal surface which is sensed by the evanescent wave. Therefore, it is a volume close to the surface that is probed. For example, when a protein layer is adsorbed on the metal surface, keeping all other parameters constant, an increase in the surface concentration occurs and the SPR angle shifts to larger values (39). The magnitude of the shift, defined as the SPR response, depends on the mean refractive index change due to the adsorption in the probed volume (a function of mass).

Utilizing SPR, biospecific interaction analysis is performed in real time in conjunction with a flow

injection system and is as sensitive as other methods such as radiolabeling, fluorometry, and chemiluminescence. In short, biospecific interaction analysis is a sensitive, nonlabile way of examining interactions between macromolecules in real time (40-42).

SPR measurements are performed on a BIAcore unit manufactured by Pharmacia Biosensor AB (Uppsala, Sweden). Sensor Chips CM5, Surfactant P20, and amine coupling kit (EDC, NHS, and ethanolamine hydrochloride) were purchased from Pharmacia Biosensor AB.

Immobilization of PSTAIR and C-terminus antibodies via amine coupling was performed according to the general procedure recommended by the manufacturer. Briefly, the instrument was equilibrated with HBS buffer (10 mM HEPES, 150 mM NaCl, 0.05% surfactant P20, pH 7.4, and filtered with a 0.22 micron filter), then the following series of injections were made using the autosampler incorporated into the BIAcore unit:

(1) equal volumes of EDC (0.1 M in water) and NHS (0.1 M in water) were mixed and 35 μL injected to activate the carboxymethylated surface;

(2) ligand (35 μL, 50μg/mL in 10 mM sodium acetate pH 4.5) was then injected;

(3) the remaining NHS-esters on the surface were then deactivated with ethanolamine (35 μL, 1 M in water, pH 8.5);

(4) noncovalently bound material was then washed from the surface with hydrochloric acid (15 μL. 20 mM). Immobilizations were executed with a continuous flow of HBS at a flow rate of 5μL/min.

A typical sensorgram produced on immobilization of anti-cdc2 C-terminus is depicted in Figure 7. Time required for immobilization is approximately 30 min.

BIAcore assay for tyrosylphosphorylation of cycline dependent kinase (CDK)-Each binding/regeneration cycle is performed with a constant flow of HBS of 3 μL/min. Hepatic S-9 fractions of rats dosed with pirnixic acid or vehicle alone are diluted to a concentration of 1.5 mg protein/mL into exhausted FB-2 tissue culture supernatant liquid and incubated overnight at 4°C with anti-phosphotyrosine antibody. This equilibrated mixture (40 μL) is then injected over the immobilized PSTAIR and C-terminus antibodies and binding is recorded in RU. Binding is directly proportional to the amount of tyrosylphosphorylated protein interacting with the anti-PSTAIR or anti-C Terminus antibodies.

Interpretation of results

Immunoblots - For scans of immunoblots, a change in phosphotyrosylprotein content of $p34^{cdc2}$ kinase greater than 40 percent was considered biologically meaningful.

BIAcore assay- Research on the cell cycle has shown that the concentration of cdc2 kinase remains constant and that tyrosine phosphorylation can be utilized as a marker of cells that are preparing to enter the M phase of the cell cycle (43-48). Therefore, increased binding indicate increased tyrosylphosphorylation of cdc2 kinase, thus more cells are in the process of preparing to enter mitosis. Treatment effects from BIAcore analyses are considered significant when the instrument response of the treatment group is outside the upper bounds of the population 95 percent confidence interval ($t_{(5)(0.95)} = 2.015$ times the

standard deviation of the RU response of the control animals).

Results

Immunoblotting analysis - As seen in Figure 8, seven proteins exhibited an increased tyrosine phosphorylation in response to the administration of pirnixic acid. A 6.24-fold increase was noted in pp69, while the greatest relative difference in peak height was seen with a 13.16-fold increase in pp33. Five phosphotyrosylproteins also evidenced a decrease in quantity. These were pp84, pp61, pp43, pp34 and pp23. Figure 9 depicts the scanning results and Figure 10 shows the quantification of the CDK at 33 kDa. Results indicate that the administration of five, twice-daily doses of pirnixic acid (50 mg/kg each dose) produces enhanced tyrosylphosphorylation of the CDK compared to control animals, which exhibit no tyrosylphosphorylation of CDK at 33 kDa. Each group on the graph represents the average of two rats. Error bars in this figure represent the 10 percent coefficient of variation in the quantification of density.

BIAcore (SPR) - Hepatic S-9 samples from rats treated with pirnixic acid produced greater binding to both anti-PSTAIR or anti-C-terminus antibodies than hepatic S-9 samples from vehicle control rats (Figure 11). This increased binding exhibited by the hepatic S-9 of test animals is due to enhanced tyrosylphosphorylation of cdc2 kinase (CDK). Figure 12 is a summary bar graph depicting BIAcore® quantification of the interaction of tyrosylphosphorylated cyclin dependent kinases (CDK) with anti-CDK polyclonal antibodies (PSTAIR and C-terminus)

from control and pirnixic acid-treated rats. Error bars represent standard deviations of n = 6 (anti-PSTAIR) and n = 8 (anti-C-terminus) control rats. RU value for pirnixic acid-treated rats represents the mean of 2 animals. The treatment of rats with 50 mg pirnixic acid/kg twice a day for 5 days results in enhanced tyrosylphosphorylation of CDK ($p34^{cdc2}$ kinase) compared to control rats.

EXAMPLE 3

Enhanced tyrosylphosphorylation of $p34^{cdc2}$ kinase in an hepatic cytosol preparation (S-9) from young male rats 24 hours following administration of the nongenotoxic carcinogen diethylhexylphthalate.

Summary

$p34^{cdc2}$ is the serine/threonine kinase subunit of M-phase promoting factor (MPF) (29-31). The regulation of $p34^{cdc2}$ tyrosine phosphorylation status is considered the control mechanism for entry into G_1 from G_0, the START signal, and also from G_2 to M, the initiation of mitosis. It is demonstrated that twice daily doses of 500 mg diethylhexylphthalate/kg of body weight for 5 days to young, male rats increases the extent of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase compared to corn oil treated controls. These results indicate that the proliferative stimulus of the nongenotoxic carcinogen diethylhexylphthalate may be quantified as an increase in hepatic $p34^{cdc2}$ kinase tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase can serve to indicate the capacity of chemicals that are termed peroxisome

proliferators to function *in vivo* as a nongenotoxic carcinogen.

Materials and Methods

Chemicals: Diethylhexylphthalate (DEHP) [CAS 117-81-7] was purchased from Fluka Chemicals (Ronkonkoma, NY). Anti-phosphotyrosine monoclonal, anti-PSTAIR (CDK), and anti-p34^{cdc2} kinase C-terminus polyclonal antibodies were obtained from UBI (Lake Placid, NY). Bicinchoninic acid was obtained from Pierce (Rockford, IL). Molecular weight standards were supplied through BioRad (Melville, NY). All other chemicals were purchased from Sigma (St. Louis, MO) and were of the highest purity available.

Animals and dosing: Rats are purchased and handled as described in Example 2.

After a wk of acclimation to new surroundings, treatments are begun. The treatment consists of twice daily doses of DEHP administered by oral gavage. The DEHP is dissolved in corn oil. Sham-treated animals are given an equal volume of plain corn oil. Doses are adjusted daily on the basis of weight. The volume of corn oil is generally on the order of 2 mL/rat throughout the treatment period. The second dose is given between the h of 13:00-16:00, approximately 6 h after the first dose given between the h of 7:00 - 10:00. The DEHP is administered for 5 days at a dose of 500 mg/kg twice a day. Rats are anesthetized and livers are prepared as described in Example 2.

Gel electrophoresis and immunoblotting with anti-phosphotyrosine: These procedures are carried out

essentially as described in Example 1 except that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 2.

Real-time quantification of total tyrosylphosphorylated $p34^{cdc2}$ kinase and interpretation of the results: These procedures are performed as described in Example 2.

Results

Immunoblotting analysis - Six phosphotyrosylproteins are shown to increase with the administration of DEHP (Figures 13 and 14). The range of relative increase is 1.48 to 4.19-fold. A decrease in pp31 and pp28 is also observed. Figure 15 depicts the quantification of the results of the scanning densitometry. The cyclin dependent kinase (CDK) quantified from the anti-phosphotyrosine immunoblot is at 34 kDa. Results indicate that the administration of five, twice-daily doses of DEHP (500 mg/kg each dose) produces enhanced tyrosylphosphorylation of the CDK compared to control animals, which exhibit no tyrosylphosphorylation of CDK at 34 kDa. Each group on the graph represents the average of two rats. Error bars represent the 10 percent coefficient of variation in the quantification of density.

BIAcore (SPR) - Hepatic S-9 samples from rats treated with DEHP produce greater binding to both anti-

PSTAIR or anti-C-terminus antibodies than hepatic S-9 samples from vehicle control rats (Figure 16). This increase in binding by the hepatic S-9 of DEHP-treated animals is due to enhanced tyrosylphosphorylation of cdc2 kinase (CDK). Figure 16 is a summary bar graph depicting BIAcore® quantification of the interaction of tyrosylphosphorylated cyclin dependent kinases (CDK) with anti-CDK polyclonal antibodies (PSTAIR and C-terminus) from control and DEHP-treated rats. Error bars represent standard deviations of n = 6 (anti-PSTAIR) and n = 8 (anti-C-terminus) control rats. RU value for DEHP-treated rats represents the mean of 2 animals. The treatment of rats with 500 mg DEHP/kg twice a day for 5 days results in enhanced tyrosylphosphorylation of CDK ($p34^{cdc2}$ kinase) compared to control rats.

EXAMPLE 4

The genotoxic carcinogen diethylnitrosamine does not enhanced tyrosylphosphorylation of $p34^{cdc2}$ kinase in an hepatic cytosol preparation (S-9) from young male rats 24 hours following administration.

Summary

$p34^{cdc2}$ is the serine/threonine kinase subunit of M-phase promoting factor (MPF) (29-31). The regulation of $p34^{cdc2}$ tyrosine phosphorylation status is considered the control mechanism for entry into G_1 from G_0, the START signal, and also from G_2 to M, the initiation of mitosis. It is demonstrated that twice daily doses of 500 mg diethylnitrosamine/kg of body weight for 5 days to young, male rats did not affect the extent of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase compared to corn oil treated controls. These results indicate that the early *in vivo* effects of the genotoxic carcinogen diethylnitrosamine can not be quantified

through a change in hepatic $p34^{cdc2}$ kinase tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase is specific for nongenotoxic carcinogens.

Materials and Methods

Chemicals: Diethylnitrosamine (DEN) [CAS 55-18-5] was purchased from Fluka Chemicals (Ronkonkoma, NY). Anti-phosphotyrosine monoclonal, anti-PSTAIR (CDK), and anti-$p34^{cdc2}$ kinase C-terminus polyclonal antibodies were obtained from UBI (Lake Placid, NY). Bicinchoninic acid was obtained from Pierce (Rockford, IL). Molecular weight standards were supplied through BioRad (Melville, NY). All other chemicals were purchased from Sigma (St. Louis, MO) and were of the highest purity available.

Animals and dosing: Animals are purchased and handled as described in Example 2. After a wk of acclimation to new surroundings, treatments are begun. The treatment consists of twice daily doses of DEN administered by oral gavage. The DEN is dissolved in corn oil. Sham-treated animals are given an equal volume of plain corn oil. Doses are adjusted daily on the basis of weight. The volume of corn oil is generally on the order of 2 mL/rat throughout the treatment period. The second dose is given between the h of 13:00-16:00, approximately 6 h after the first dose given between the h of 7:00 - 10:00. The DEN is administered for 5 days at a dose of 500 mg/kg twice a day. Rats are anesthetized and livers are prepared as described in Example 2.

Gel electrophoresis and immunoblotting with anti-phosphotyrosine: These procedures are carried out as described in Example 1 except that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Real-time quantification of total tyrosylphosphorylated $p34^{cdc2}$ kinase and interpretation of the results: These procedures are performed as described in Example 2.

Results

Immunoblotting analysis - Administration of DEN to young male rats did not produce any increases in phosphotyrosylproteins (Figures 17 and 18). A 61% decrease in pp22 is observed. Figure 19 is a bar graph depicting the quantification of the results of the scanning densitometry. The band quantified from the anti-phosphotyrosine immunoblot is at 34 kDa. Results indicate that the administration of five, twice-daily doses of DEN (500 mg/kg each dose) produces no enhanced tyrosylphosphorylation of the p34 compared to control animals. Each group on the graph represents the average of two rats. Error bars represent the 10 percent coefficient of variation in the quantification of density.

BIAcore (SPR) - Hepatic S-9 samples from rats treated with DEN produce no greater binding to anti-PSTAIR or anti-C-terminus antibodies than hepatic S-9 samples from vehicle control rats. Figure 20 is a

summary bar graph depicting BIAcore® quantification of the interaction of tyrosylphosphorylated cyclin dependent kinases (CDK) with anti-CDK polyclonal antibodies (PSTAIR and C-terminus) from control and DEN-treated rats. Error bars represent standard deviations of n = 6 (anti-PSTAIR) and n = 8 (anti-C Terminus) control rats. RU value for DEN-treated rats represents the mean of 2 animals. Results indicate that the treatment of rats with 500 mg DEN/kg twice a day for 5 days produces no enhanced tyrosylphosphorylation of CDK ($p34^{cdc2}$ kinase) compared to control rats.

EXAMPLE 5

Enhanced tyrosylphosphorylation of p34 in an hepatic cytosol preparation (S-9) from female Beagle dogs following administration of the nongenotoxic carcinogen Aroclor® polychlorinated biphenyls for eleven and one-half weeks

Summary

It is demonstrated that daily doses of 0.6, 0.8, 4-8, or 5-10 mg /kg of body weight for 11.5 weeks to 2-year old, female Beagle dogs enhances the tyrosine phosphorylation status of an hepatic p34 compared to corn oil treated controls. These results indicate that the early *in vivo* effects of the nongenotoxic carcinogen Aroclor® polychlorinated biphenyls can be quantified through a change in hepatic p34 tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of hepatic p34 is specific for nongenotoxic carcinogens.

Materials and Methods

Chemicals: Aroclor®1254 polychlorinated biphenyls (PCBs) is purchased from AccuStandard, Inc.

(New Haven, CT). Anti-phosphotyrosine monoclonal antibody is obtained from UBI (Lake Placid, NY). Bicinchoninic acid is obtained from Pierce (Rockford, IL). Molecular weight standards are supplied through BioRad (Melville, NY). All other chemicals were purchased from Sigma (St. Louis, MO) or stated suppliers and were of the highest purity available.

Animals and dosing: Five, purebred, 2-year old, female beagle dogs, obtained from Norwich Pharmaceutical (Norwich, NY), are used in this study. All dogs were fully vaccinated, dewormed and specific pathogen free (SPF) for at least 30 days prior to the initiation of the experiment. They are maintained indoors and individually housed according to Public Health service guidelines (NIH publication No. 85-23). At the beginning of the study the dogs weigh between 8.7 and 12.2 kg. Physical observations of the dogs are made daily during the 11.5-wk dosing period of the study.

Each dog is administered either corn oil (controls) or Aroclor® PCBS at 0.6, 0.8, 4 or 5 mg/kg-day for seven wk. From seven to 11.5 wk, the 4 mg/kg-day dose and the 5 mg/kg-day dose are increased to 8 and 10 mg/kg-day, respectively. The corn oil, as well as test material, is administered in a cube of agarose concealed in a small ball of canned dog food. After consumption of the meatball, the dogs are immediately fed their daily caloric requirement of canned food.

Dogs were sacrificed using 2mL/kg of Fatal Plus (Vortech Pharmaceutical Company, Dearborne, MI). Hepatic S-9 fractions were prepared as previously described in Example 1.

Gel electrophoresis and immunoblotting with anti-phosphotyrosine: These procedures are carried out as described in Example 1 with the exception that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Results

The daily administration of Aroclor® polychlorinated biphenyls for a period of 11.5 wk results in enhanced tyrosylphosphorylation of a protein migrating at 34 kDa at all doses compared to the control dog. Figure 21 depicts the anti-phosphotyrosine immunoblot of dog hepatic S-9 protein separated using 11% SDS-PAGE gels for control and Aroclor® polychlorinated biphenyls-treated dogs. Lanes 1,2,3,4, and 5 are control, 0.6, 0.8, 4-8, and 5-10 mg Aroclor®/kg-day, respectively. The scanning densitometry of a single band at p34 of the anti-phosphotyrosine immunoblot is presented in Figure 22. Quantification of the scanning densitometry of p34 is presented in Figure 23 as a bar graph. Each bar on the graph represents the single result of scanning an immunoblot produced from the hepatic S-9 of one dog. Error bars represent the 10 percent coefficient of variation in the quantification of density.

In Vitro Experiments

EXAMPLE 6

Enhanced tyrosylphosphorylation of p34/p33 (putative CDK) in 3T3 cell lysates 24 hours following exposure to the nongenotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Summary

It is demonstrated that exposure of 3T3 cells to 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin for 24 h in a low serum media enhances the tyrosine phosphorylation status of two cell lysate proteins, p34 and p33, compared to dimethylsulfoxide-treated controls. These results indicate that the early *in vitro* effects of the nongenotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin can be quantified through a change in cellular p34/p33 tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of p34/p33 is specific for nongenotoxic carcinogens.

Materials and Methods

Chemicals: 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is purchased from AccuStandard, Inc. (New Haven, CT). Anti-phosphotyrosine monoclonal antibody is obtained from UBI (Lake Placid, NY). Bicinchoninic acid is obtained from Pierce (Rockford, IL). Molecular weight standards are supplied through BioRad (Melville, NY). All other chemicals were purchased from Sigma (St. Louis, MO) or stated suppliers and were of the highest purity available.

Tissue culture cells, culture conditions and dosing: 3T3 cells (ATCC CCL-92) are purchased from American Type

Culture Collection (Bethesda, MD). These cells are maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco cat. #430-2100) supplemented with 10% Fetal bovine serum-heat inactivated (FBS-HI) (Intergen, Purchase, NY). For experimental purposes, the cells are plated in 100 mm x 20 mm tissue culture dishes containing 10 mL of the above maintenance medium. The plates are placed in an incubator set at 37°C, 5% CO_2, 95% humidity, until they reach confluence (contact inhibited). At this point all the plates are then washed 2x with 5 mL of Dulbecco's calcium and magnesium-free phosphate buffered saline (CMF-PBS). Four plates are then fed 10 mL of DMEM + 10% FBS-HI and all the other plates are fed 10 mL of DMEM + 0.5% FBS-HI and incubated for 48 h in the above environmental conditions.

After the 48 h incubation period, the medium from the low-serum group (0.5% FBS-HI) was aseptically harvested and allocated into separate tubes containing 40 mL each (to provide 10 mL/plate for 4 plates per treatment). The following concentrations and reagents are added to the appropriate tubes (4 plates/treatment). Dimethyl sulfoxide (DMSO) is used as the diluent for TCDD.

10 mL of DMEM + 20% FBS-HI + 0.1% DMSO (positive control)

10 mL of DMEM + 0.5% FBS-HI + 0.1% DMSO

10 mL of DMEM + 0.5% FBS-HI + 10 nM TCDD

All plates were returned to the incubator for 24 h at the environmental conditions listed above. After the 24 h incubation period, the cells are harvested using the harvesting procedure described.

Gel electrophoresis and immunoblotting with anti-phosphotyrosin : These procedures are carried out as described in Example 1 with the exception that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Results

Exposure of 3T3 cells to 10 nM TCDD for 24 h results in an increase in tyrosylphosphorylation of p34 and p33 of 67 and 32%, respectively, compared to the vehicle control. The anti-phosphotyrosine immunoblot of 3T3 cell lysate protein separated using an 11% SDS-PAGE gel for 3T3 cells exposed to 10 nM TCDD is presented in Figure 24. Results of scanning the control and TCDD-treated lanes are presented in Figure 25; bolded peaks indicate p34 and p33 tyrosylphosphophoproteins. In Figure 26 the putative cyclin dependent kinases (p34/p33) are quantified from the anti-phosphotyrosine immunoblot. Results of serum supplementation (c.f. immunoblot in Figure 24, scan results not depicted in Figure 25) indicate enhanced tyrosylphosphorylation of p34/p33. This result would be expected if the pp34/pp33 are cyclin dependent kinases, since the serum supplemented media provide growth factor that stimulate the cells to mitosis and this stimulus is mediated through the CDK.

EXAMPLE 7

Enhanced tyrosylphosphorylation of p34/p33 (putative CDK) in 3T3 cell lysates 24 hours following exposure to the tumor promotor 12-O-tetra-decanoylphorbol-13-acetate.

Summary

It is demonstrated that exposure of 3T3 cells to 12-O-tetra-decanoylphorbol-13-acetate for 24 h in a low-serum media enhances the tyrosine phosphorylation status of two cell lysate proteins, p34 and p33, compared to dimethylsulfoxide-treated controls. These results indicate that the early *in vitro* effects of the tumor promotor 12-O-tetra-decanoylphorbol-13-acetate can be quantified through a change in cellular p34/p33 tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of p34/p33 is specific to a mechanism relating to the process of nongenotoxic carcinogenesis.

Materials and Methods

Chemicals: 2-O-Tetra-decanoylphorbol-13-acetate (TPA) is purchased from ChemSyn Science Labs (Lenexa, KY). Anti-phosphotyrosine monoclonal antibody is obtained from UBI (Lake Placid, NY). Bicinchoninic acid is obtained from Pierce (Rockford, IL). Molecular weight standards are supplied through BioRad (Melville, NY). All other chemicals were purchased from Sigma (St. Louis, MO) or stated suppliers and were of the highest purity available.

Tissue culture cells, culture conditions and dosing: These procedures are performed as described in Example 6. The following concentrations and reagents are added to the appropriate tubes (4 plates/treatment). Dimethyl sulfoxide (DMSO) is used as the diluent for TPA.

10 mL of DMEM + 20% FBS-HI + 0.1% DMSO
10 mL of DMEM + 0.5% FBS-HI + 0.1% DMSO
10 mL of DMEM + 0.5% FBS-HI + 160 nM TPA

Gel electrophoresis and immunoblotting with anti-phosphotyrosine: These procedures are carried out as described in Example 1 except that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Results

Exposure of 3T3 cells to 160 nM TPA for 24 h results in an increase in tyrosylphosphorylation of p34 and p33 of 54 and 95%, respectively, compared to the vehicle control. The anti-phosphotyrosine immunoblot of 3T3 cell lysate protein separated using an 11% SDS-PAGE gel for 3T3 cells exposed to 10 nM TCDD is presented in Figure 27. Results of scanning the control and TCDD-treated lanes are presented in Figure 28; bolded peaks indicate p34 and p33 tyrosylphosphosphoproteins. In Figure 29 the putative cyclin dependent kinases (p34/p33) are quantified from the anti-phosphotyrosine immunoblot. Results of serum supplementation (c.f. immunoblot in Figure 27, scan results not depicted in Figure 28)

indicate enhanced tyrosylphosphorylation of p34/p33. This result would be expected if the pp34/pp33 are cyclin dependent kinases, since the serum supplemented media provide growth factor that stimulate the cells to mitosis and this stimulus is be mediated through the CDK.

EXAMPLE 8

Enhanced tyrosylphosphorylation of p34/p33 in BNL CL.2 cell lysates 24 hours following exposure to the nongenotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Summary

It is demonstrated that exposure of BNL CL.2 cells to 0.1, 1, 10 or 100 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin for 24 h in a low serum media enhances the tyrosine phosphorylation status of two cell lysate proteins, p34 and p33, compared to dimethylsulfoxide-treated controls. These results indicate that the early *in vitro* effects of the nongenotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin can be quantified through a change in cellular p34/p33 tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of p34/p33 is specific for nongenotoxic carcinogens.

Materials and Methods

Chemicals: This section is as previously described in Example 6.

Tissue culture cells, culture conditions and dosing: BNL CL.2 cells (ATCC TIB73) are purchased from American Type Culture Collection (Bethesda, MD). These cells are representative of normal mouse hepatocytes.

All other procedures were performed as detailed in Example 6.

The following concentrations and reagents are added to the appropriate tubes (4 plates/treatment). Dimethyl sulfoxide (DMSO) is used as the diluent for TCDD.

10 mL of DMEM + 20% FBS-HI + 0.1% DMSO (positive control)
10 mL of DMEM + 0.5% FBS-HI + 0.1% DMSO
10 mL of DMEM + 0.5% FBS-HI + 0.1 nM TCDD
10 mL of DMEM + 0.5% FBS-HI + 1.0 nM TCDD
10 mL of DMEM + 0.5% FBS-HI + 10 nM TCDD
10 mL of DMEM + 0.5% FBS-HI + 100 nM TCDD

All plates were returned to the incubator for 24 h at the environmental conditions listed above. After the 24 h incubation period, the cells are harvested using the harvesting procedure described.

Gel electrophoresis and immunoblotting with anti-phosphotyrosine: These procedures are carried out as described in Example 1 except that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Results

Exposure of BNL CL2 cells to 0.1, 1, 10 or 100 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for 24 h results in a simialr increase in tyrosylphosphorylation of p34, averaging 180% of the vehicle control over all

test concentrations of TCDD. Twenty percent serum supplementation results in an increase of tyrosylphosphorylation of p34 of 229% of the vehicle control. Vehicle controls at 0.5% serum supplementation exhibit no tyrosylphosphorylation at p33, while TCDD exposure at the four concentrations enhances tyrosylphosphorylation of this putative CDK to 0.9, 2.0, 2,0 and 1.9 density units, respectively. The increases in tyrosylphosphorylation of p33 by TCDD are 3.4 times the p33 tyrosine phosphorylation produced by 20% serum supplementation. The anti-phosphotyrosine immunoblot of BNL CL.2 cell lysate protein separated using an 11% SDS-PAGE gel for BNL CL.2 cells exposed to the four concentrations of TCDD is presented in Figure 30. Results of scanning the control and TCDD-treated lanes are presented in Figure 31; the represented peaks are p34 and p33 tyrosylphosphophoproteins. In Figure 32 the putative cyclin dependent kinases (p34/p33) are quantified from the anti-phosphotyrosine immunoblot.

EXAMPLE 9

Enhanced tyrosylphosphorylation of p34/p33 in BNL CL.2 cell lysates 24 hours following exposure to the nongenotoxic carcinogen pirnixic acid.

Summary

It is demonstrated that exposure of BNL CL.2 cells to 1, 10 or 100 nM pirnixic acid for 24 h in a low serum media enhances the tyrosine phosphorylation status of two cell lysate proteins, p34 and p33, compared to dimethylsulfoxide-treated controls. These results indicate that the early *in vitro* effects of the nongenotoxic carcinogen pirnixic acid can be quantified

through a change in cellular p34/p33 tyrosylphosphorylation and that stimulation of tyrosylphosphorylation of p34/p33 is specific for nongenotoxic carcinogens.

Materials and Methods

Chemicals: This section is as previously described in Example 7.

Tissue culture cells, culture conditions and dosing: BNL CL.2 cells (ATCC TIB73) are purchased from American Type Culture Collection (Bethesda, MD). These cells are representative of normal mouse hepatocytes. All other procedures were performed as detailed in Example 7.

The following concentrations and reagents are added to the appropriate tubes (4 plates/treatment). Dimethyl sulfoxide (DMSO) is used as the diluent for TCDD.

10 mL of DMEM + 20% FBS-HI + 0.1% DMSO (positive control)

10 mL of DMEM + 0.5% FBS-HI + 0.1% DMSO

10 mL of DMEM + 0.5% FBS-HI + 1 nM pirnixic acid

10 mL of DMEM + 0.5% FBS-HI + 10 nM pirnixic acid

10 mL of DMEM + 0.5% FBS-HI + 100 nM pirnixic acid

10 mL of DMEM + 0.5% FBS-HI + 1000 nM pirnixic acid

All plates were returned to the incubator for 24 h at the environmental conditions listed above. After the 24 h incubation period, the cells are harvested using the harvesting procedure described.

Gel electrophoresis and immunoblotting with anti-phosphotyrosine: These procedures are carried out as described in Example 1 except that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Results

Exposure of BNL CL2 cells to pirnixic acid for 24 h results in increases in tyrosylphosphorylation of p34 relative to the vehicle control for the 1 and 10 nM concentrations, 96 and 58% increases, respectively. At 100 nM pirnixic acid the tyrosylphosphorylation of p34 is similar to the vehicle control, while at 1000 nM tyrosine phosphorylation of p34 is depressed 60% from the vehicle control. Twenty percent serum supplementation results in an increase of tyrosylphosphorylation of p34 of 229%, relative to the vehicle control. The 5% serum supplementation control exhibits no tyrosylphosphorylation at p33, while pirnixic acid exposure enhances tyrosylphosphorylation of this putative CDK to 2.0, 2.5 and 0.5 density units, respectively, at the 1, 10, and 100 nM concentrations. The increases in tyrosylphosphorylation of p33 by pirnixic acid at 1 and

10 nM are roughly 4 times the p33 tyrosine phosphorylation produced by 20% serum supplementation.

The anti-phosphotyrosine immunoblot of BNL CL.2 cell lysate protein separated using an 11% SDS-PAGE gel for BNL CL.2 cells exposed to the four concentrations of pirnixic acid is presented in Figure 33. Results of scanning the control and TCDD-treated lanes are presented in Figure 34; the represented peaks are p34 and p33 tyrosylphosphophoproteins. In Figure 35 the putative cyclin dependent kinases (p34/p33) are quantified from the anti-phosphotyrosine immunoblot.

EXAMPLE 10

Use of a microtiter assay for the assessment of enhanced tyrosylphosphorylation of cyclin-dependent kinases (CDK) or $p34^{cdc2}$ kinase in hepatic, pulmonary and renal cytosol (S-9) preparations from C57BL/6J female mice administered 2,3,78-tetrachlorodibenzo-p-dioxin for 90 days

Summary

The regulation of the tyroslyphosphorylaltion status of the cytosolic cyclin dependent kinases (CDK) is considered the control mechanism for the entry into G_1 from G_0, the START signal, and also for the movement of the cell from G_2 to M, the initiation of mitosis. A microtiter kit is described that allows for the demonstration of enhanced tyrosylphosphorylation of hepatic CDK as well as $p34^{cdc2}$ kinase following the daily administration of 0.25, 0.5, 1 or 2 ng 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)/kg to young, female mice for 90 days. It is also demonstrated that the microtiter kit may be used to assay for enhanced tyrosylphosphorylation of CDK in extrahepatic tissues and thus allow for the identification of the most sensitive responding tissue.

Materials and Methods

Materials and Chemicals:

Immobilon 2 microtiter plates	Dynatech (Shantilly, VA)
Anti-PSTAIR polyclonal antibody	UBI (Lake Placid, NY)
Anti-C-terminus polyclonal antibody	UBI (Lake Placid, NY)
Anti-phosphotyrosine monoclonal antibody	UBI (Lake Placid, NY)
Peroxidase-labeled rabbit anti-primary antibody	BioRad (Melville, NY)
BSA (bovine serum albumin)	[Sigma #A-3350]
Triton X-100	[Sigma #X-100]
EGTA (ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid)	[Sigma #E-4378]
PMSF (phenylmethylsulfonyl fluoride)	[Sigma #P-7626]
Leupeptin	[Sigma #L-2884]
Soy bean trypsin inhibitor	[Sigma #T-9003]
N-Tosyl-L-phenylalanine chloromethyl ketone	[Sigma #T-4376]
Sodium fluoride	[Sigma #S-6521]
β-Glycerophosphate	[Sigma #G-6626]
Paranitrophenyl phosphate	[Sigma #104-0]
Sodium orthovanadate	[Sigma #S-6508]
DTT (dithiothreitol)	[Sigma #D-0632]
$MgCl_2$	
ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonicacid) diammonium salt)	[Sigma #A-1888]
H_2O_2 (hydrogen peroxide)	[Sigma #H-1009]
TRIS	[Sigma (St. Louis, MO)]
Na Carbonate	[Sigma (St. Louis, MO)]
2,3,7,8-tetrachlorodibenzo-p-dioxin	[AccuStandard, Inc. (New Haven, CT)]

Reagents:

A. *Sodium carbonate buffer*; 0.1M, pH 9.6

a) Mix 71.3 ml of 1M $NaHCO_3$ and 28 ml of 1M Na_2CO_3.

b) Add 800 ml ddH_2O.

c) Adjust pH to 9.6 and Qs to 1 l.

B. *10X Phosphate buffered saline*; 0.15M, pH 7.2

a) NaCl, 80.0 g/l.

b) KCl, 2.0 g/l.

c) Na_2HPO_4, 11.5 g/l.

d) NaH_2PO_4, 2.0 g/l.

C. *Blocking buffer*; PBS with 3% BSA

a) 1X PBS with 3 g BSA per 100 ml.

D. *Washing buffer*; PBS with 0.2% Triton X-100

a) 1X PBS with 0.2 ml of Triton X-100 per ml.

E. *Prep Buffer*; 25mM Tris-HCl, pH 8.0 with 10mM $MgCl_2$, 15mM EGTA, 0.1% Triton X-100, 0.1mM PMSF, 0.1mM Na fluoride, 60mM β-glycerophosphate, 15mM paranitrophenylphosphate, 0.1mM Na orthovanidate, 1μg/ml leupeptin, 10μg/ml soybean trypsin inhibitor, 1μg/ml aprotinin, and 10μg/ml tosyl phenylalanine.

F. *Assay buffer*; 50mM Tris-HCl, pH 7.4 with 10mM $MgCl_2$, 1mM DTT, and all inhibitors of phosphatases and proteases contained in *Prep buffer*.

G. *Citrate buffer*;

a) Add 9.6 g Citric acid (MW 192.12) to 950 ml ddH_2O.

b) Adjust pH to 4.0 with 5M NaOH and store at 4°C.

H. *ABTS stock solution*;

a) 0.5487 g ABTS to 25 ml with double distilled H_2O and store at 4°C.

I. *ABTS substrate*; 0.4mM ABTS

a) 0.05 ml ABTS

b) 0.02 ml diluted H_2O_2 (0.5M)

c) 5.0 ml citrate buffer

Animals and dosing: Four to six-wk old, female C57BL/6J mice are obtained from Harton Sprague Dawley (Indianapolis, IN). The mice are fed Prolab RMH 1000 (Agway, Cortland, NY) and receive tap water *ad libitum*. All mice are housed three per cage and maintained on a photoperiod of 12 h. Mice are administered TCDD in corn oil at 0, 0.25, 0.5, 1, or 2 ng/kg by oral gavage daily for a period of 90 days. Ten mice are treated at each dose and the volume of the dose is approximately 0.1 mL per mouse.

Procedure:

Plate preparation:

1. 100 µl of anti-PSTAIR or anti-C-terminus antibody at a concentration of 10 µg/mL in of 0.1M *Na carbonate buffer* pH 9.6 is added to the wells of a microtiter plate and incubated overnight at 4°C. These are the capture antibodies and will retain all CDK or $p34^{cdc2}$ kinase, respectively.
2. Wash plates 3x with *washing buffer* by filling the wells, allowing them to sit for two minutes, and inverting and shaking them. This step removes all
3. Block plates for two hours at room temp by filling the wells with *blocking buffer*. The plates can be washed 1x with *washing buffer* and stored for several weeks at 4°C.
4. Wash fresh plated 3x or stored plates 2x with *washing buffer* prior to use.

Sample preparation:

All preparation procedures are performed on individual or pooled hepatic, pulmonary or renal samples. Preparation and -80°C storage of tissue S-9 fractions is performed exactly as previously described in the scientific literature (32). This procedure involves killing the mouse by cervical dislocation, removing the liver, lung or kidney sample and homogenizing the tissue in three volumes of *Prep buffer*. This tissue homogenate is centrifuged at 9,000 x g for 20 min at 4°C. The resulting supernatant fraction, termed the S-9, is decanted into 1.5 mL plastic, conical tubes, frozen in a dry ice/ethanol bath and stored at -80°C until the microtiter assay can be performed.

Assay:

1. 200 µg of sample tissue protein is diluted in *Prep buffer* and mixed 1:1 with *blocking buffer*.
2. This is added to the wells of a prepared plate and incubated for 5 hr at 4°C with slow constant shaking.
3. Plates are washed 3x with *washing buffer* and 1x with *assay buffer*.
4. 200 µl of primary (anti-phosphotyrosine) antibody at a dilution of 1:1000 in blocking buffer is added to each well and incubated for 2 hr at 4°C.
5. Plates are washed 3x with washing buffer.
6. 200 µl of peroxidase-conjugated (anti-mouse) secondary antibody at a dilution of 1:3000 in blocking buffer is added to each well and incubated for 1 hr at 4°C.
7. Wash plates 3x with washing buffer.

8. Add 200 µl of ABTS solution and read once a minute for 10 min in kinetics mode (Biotek EL312) at 415 nm.

Interpretation of results

Microtiter assay - The anti-PSTAIR or anti-C-terminus antibody will, respectively, capture all CDK or $p34^{cdc2}$ kinase present in the tissue S-9 fraction in the microtiter well. The anti-phosphotyrosine antibody quantifies the extent of tyrosylphosphorylation of the total CDK or p34cdc2 kinase. This quantification represents the extent to which the cells from the sampled tissue have been signaled to exit the Go stage of the cell cycle (index of proliferative signaling) by exposure to the test chemical. The current state of knowledge in the role of the cyclin dependent kinases in controlling the cell cycle (43-48) does not allow for an absolute determination as to the extent of CDK tyrosylphosphorylation relating to the strength of the proliferative signal. The fact that molecules other than peptide-like growth factors have the ability to enhance the tyrosylphosphorylation status of the CDK has not been reported in the literature. Therefore, interpretation of the capacity of a test chemical to direct the cell toward mitosis relies on a comparison to a control group treated only with the vehicle. A test chemical is considered positive for the capacity to function as a nongenotoxic carcinogen when the extent of CDK or $p34^{cdc2}$ kinase tyrosylphophorylation is statistically greater ($p < 0.05$) than a concurrent control.

Results

Microtiter assay - As seen in Figure 36, the dosing of C57BL/6J female mice with 0, 0.25. 0.5, 1 or 2 ng

TCDD/kg-day (A, B, C and D, respectively) for 90 days results in enhanced tyrosylphosphorylation of hepatic CDK but not pulmonary or renal CDK. This identifies the target tissue for the cellular proliferative effects of TCDD as the liver. Maximal increase in tyrosylphoshorylation of hepatic CDK is observed at the 0.5 ng TCDD/kg-day dose regimen. Results for the tryosylphosphorylation of $p34^{cdc2}$ kinase are similar (Figure 37), although the absolute increase observed is lower. This is due to the fact that p34cdc2 kinase represents only one of several possible CDK in the cytosol that function to regulate cell replecation.

EXAMPLE 11

Use of a microtiter assay for the assessment of enhanced expression of cyclin-dependent kinases (CDK) or $p34^{cdc2}$ kinase in hepatic cytosol (S-9) preparations from young male rats 1, 2, or 3 days following the administration of the nongenotoxic carcinogen pirinixic acid (WY14,643)

Summary

This example demonstrates of the utility of the assay for the quantification of CDK response elicited by a test chemical *in vivo* following an exposure period of any length and a description of a kit to perform the assay.

It is observed that the administration of the nongenotoxic carcinogen pirinixic acid to young, male rats results in the enhanced expression of total cytosolic cyclin-dependent kinases (CDK). A microtiter kit is described that allows for the demonstration of enhanced expression of hepatic CDK as well as $p34^{cdc2}$ kinase following a single dose of 50 mg pirinixic acid.

Materials and Methods

Materials and Chemicals:

Immobilon 2 microtiter plates	Dynatech (Shantilly, VA)
Anti-C-terminus cdc2 polyclonal antibody	UBI (Lake Placid, NY)
Anti-PSTAIR	UBI (Lake Placid, NY)
Peroxidase-labeled rabbit anti-primary antibody	BioRad (Melville, NY)
BSA (bovine serum albumin)	[Sigma #A-3350]
Triton X-100	[Sigma #X-100]
EGTA (ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid)	[Sigma #E-4378]
PMSF (phenylmethylsulfonyl fluoride)	[Sigma #P-7626]
Leupeptin	[Sigma #L-2884]
Soy bean trypsin inhibitor	[Sigma #T-9003]
N-Tosyl-L-phenylalanine chloromethyl ketone	[Sigma #T-4376]
Sodium fluoride	[Sigma #S-6521]
β-Glycerophosphate	[Sigma #G-6626]
Paranitrophenyl phosphate	[Sigma #104-0]
Sodium orthovanadate	[Sigma #S-6508]
DTT (dithiothreitol)	[Sigma #D-0632]
$MgCl_2$	
ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt)	[Sigma #A-1888]
H_2O_2 (hydrogen peroxide)	[Sigma #H-1009]
TRIS	[Sigma (St. Louis, MO)]
Na Carbonate	[Sigma (St. Louis, MO)]
Pirinixic acid	[ChemSyn Labs (Lenexa, KY)]

Reagents:

A. *Sodium carbonate buffer*; 0.1M, pH 9.6

a) Mix 71.3 ml of 1M $NaHCO_3$ and 28 ml of 1M Na_2CO_3.

b) Add 800 ml ddH_2O.

c) Adjust pH to 9.6 and Qs to 1 l.

B. *10X Phosphate buffered saline*; 0.15M, pH 7.2

a) NaCl, 80.0 g/l.

b) KCl, 2.0 g/l.

c) Na_2HPO_4, 11.5 g/l.

d) NaH_2PO_4, 2.0 g/l.

C. *Blocking buffer*; PBS with 3% BSA

a) 1X PBS with 3 g BSA per 100 ml.

D. *Washing buffer*; PBS with 0.2% Triton X-100

a) 1X PBS with 0.2 ml of Triton X-100 per ml.

E. *Prep Buffer*; 25mM Tris-HCl, pH 8.0 with 10mM $MgCl_2$, 15mM EGTA, 0.1% Triton X-100, 0.1mM PMSF, 0.1mM Na fluoride, 60mM β-glycerophosphate, 15mM paranitrophenylphosphate, 0.1mM Na orthovanidate, 1μg/ml leupeptin, 10μg/ml soybean trypsin inhibitor, 1μg/ml aprotinin, and 10μg/ml tosyl phenylalanine.

F. *Assay buffer*; 50mM Tris-HCl, pH 7.4 with 10mM $MgCl_2$, 1mM DTT, and all inhibitors of phosphatases and proteases contained in *Prep buffer*.

G. *Citrate buffer*;

a) Add 9.6 g Citric acid (MW 192.12) to 950 ml ddH_2O.

b) Adjust pH to 4.0 with 5M NaOH and store at 4°C.

H. *ABTS stock solution*;

a) 0.5487 g ABTS to 25 ml with double distilled H_2O and store at 4°C.

I. *ABTS substrate*; 0.4mM ABTS

a) 0.05 ml ABTS

b) 0.02 ml diluted H_2O_2 (0.5M)

c) 5.0 ml citrate buffer

Animals, dosing and preparation of tissue S9: This procedure is performed as described in Example 2 except only a single 50 mg/kg dose of pirinixic acid is administered. Livers are removed from rats on postdosing days 1, 2 and 3.

Gel electrophoresis and immunoblotting with anti-cdc2 C-terminus: These procedures are carried out as described in Example 1 except that anti-cdc2 C-terminus is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Microtiter assay procedure:

Sample preparation:

All preparation procedures are performed on individual or pooled hepatic (tissue) samples. Preparation and -80°C storage of tissue S9 fractions is performed exactly as previously described in the scientific literature (32). This procedure involves killing the rat by cervical dislocation, removing and homogenizing the tissue in three volumes of *Prep buffer*. This tissue homogenate is centrifuged at 9,000 x g for 20 min at 4°C. The resulting supernatant fraction, termed the S9, is decanted into 1.5 ml plastic, conical tubes, frozen in a dry ice/ethanol bath and stored at -80°C until the microtiter assay can be performed.

Assay:

1. 50 μg of S9 tissue protein is diluted in *Prep buffer* and mixed 1:1 with *blocking buffer*.

2. This is added to the wells of a prepared plate and incubated for 5 hr at 4°C with slow constant shaking.

3. Plates are washed 3x with *washing buffer* and 1x with *assay buffer*.

4. 200 μl of primary (anti-cdc2 C-terminus) antibody at a dilution of 1:1000 in blocking buffer is added to each well and incubated for 2 hr at 4°C.

5. Plates are washed 3x with washing buffer.

6. 200 μl of peroxidase-conjugated (anti-mouse) secondary antibody at a dilution of 1:3000 in blocking buffer is added to each well and incubated for 1 hr at 4°C.

7. Wash plates 3x with washing buffer.

8. Add 200 μl of ABTS solution and read once a minute for 10 min in kinetics mode (Biotek EL312) at 415 nm.

Interpretation of results

Microtiter assay - Due to cross-reactivity with other, unidentified CDK, the anti-cdc2 C-terminus antibody will quantify the total CDK expression in the tissue. This quantification represents the extent to which the cells from the sampled tissue have been signaled to exit the G_0 stage of the cell cycle (index of proliferative signaling) by exposure to the test chemical. The current state of knowledge in the role of the cyclin dependent kinases in controlling the cell cycle (43-48) does not allow for an explanation as to the strength of the proliferative signal. The fact that molecules other than peptide-like growth factors have the ability to enhance the expression of the CDK has not been reported in the literature. Therefore, interpretation of the capacity of a test chemical to direct the cell toward replication relies on a comparison to a concurrent control group treated only with the vehicle used to administer the test chemical. A test chemical is considered positive for the capacity to function as a nongenotoxic carcinogen when the extent of CDK or $p34^{cdc2}$ kinase expression is statistically greater ($p < 0.05$) than a concurrent control.

Results

Immunoblotting with anti-cdc2 C-terminus - Figure 38 depicts the immunoblot of rat hepatic S9 protein separated using 10 to 11% SDS-PAGE gels for control (lanes 1 and 3) and WY14,643-treated rats (lanes 2 and 4). A single intensely-stained band was visible in the CDK region (32 to 35 kDa) in hepatic S9 samples obtained from rats 3 days after receiving a single dose of 50 mg WY14,643/kg.

Microtiter assay - As seen in Figure 39, the extent of CDK expression in the livers of young, male rats

receiving a single dose of 50 mg/kg of WY14,643 increases steadily during the 3-day postdosing observation period. CDK expression in control animals remains constant over the same 3-day period.

EXAMPLE 12

Enhanced expression of CDK in BNL CL.2 cell lysates 48 hours following exposure to the nongenotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Summary

This example demonstrates the utility of the assay for the quantification of CDK response elicited by a test chemical in vitro following an exposure period of 48 hours.

It is demonstrated that exposure of BNL CL.2 cells to 0.1, 1, or 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin for 48 hours in a low serum media enhances the expression of two cell lysate proteins, p34 and p33 immunoreactive with anti-cdc2 C-terminus antibody, compared to dimethylsulfoxide-treated controls. These results indicate that the early in vitro effects of the nongenotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin can be quantified through a change in celluar CDK expression and therefore that stimulation of CDK is specific for nongenotoxic carcinogens.

Materials and Methods

Chemicals: This section is as previously described in Example 6.

Tissue culture cells, culture conditions and dosing: BNL CL.2 cells (ATCC TIB73) are purchased from American Type Culture Collection (Bethesda, MD). These

cells are representative of normal mouse hepatocytes. All other procedures were performed as detailed in Example 6.

The following concentrations and reagents are added to the appropriate tubes (4 plates/treatment). Dimethyl sulfoxide (DMSO) is used as the diluent for TCDD.

10 mL of DMEM + 20% FBS-HI + 0.1% DMSO (positive control)

10 mL of DMEM + 0.5% FBS-HI + 0.1% DMSO

10 mL of DMEM + 0.5% FBS-HI + 0.1 nM TCDD

10 mL of DMEM + 0.5% FBS-HI + 1.0 nM TCDD

10 mL of DMEM + 0.5% FBS-HI + 10 nM TCDD

All plates were returned to the incubator for 48 h at the environmental conditions listed above. After the 48 h incubation period, the cells are harvested using the harvesting procedure described.

Gel electrophoresis and immunoblotting with anti-cdc2 C-terminus: These procedures are carried out as described in Example 1 except that anti-cdc2 C-terminus antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Results

Exposure of BNL CL2 cells to 0.1, 1, or 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for 48 h results in an increase in expression of anti-cdc2 C-terminus immunoreactive proteins p34 and p33 compared to the serum deprived DMSO control (Figure 40, lanes 8, 9 and 10 compared to lane 6). CDK protein expression at 10 nM TCDD was similar to that observed with serum stimulation (lane 10 compared to lane 7).

EXAMPLE 13

Testing Chemical Compounds or
Test Samples for Nongenotoxic Carcinogens

The assays systems and methods disclosed in Examples 1-12 can be used to test chemical compounds, human and animal serum, air, water, and soil environmental samples for the presence of nongenotoxic carcinogens.

The above reagents, including antibodies, with or without aliquots of the cell lines described in the Examples may be packaged in the form of kits for the testing of suspected nongenotoxic carcinogens. Equivalent reagents, antibodies or cell lines may be substituted for the ones described in the Examples. In one preferred embodiment, a panel of three cell lines are included in the test kits. The three cell lines are a murine cell line, a rat cell line and a human cell line. Cell lines which are suitable for this purpose include murine BNL-CL.2 cells, a primary rat hepatic cell line developed by Paracelsian, Inc., PRLN-RH1, and a human hepatic cell line such as Hep G2 (ATCC: HB-8065).

Tissue samples, cells, and cell lysates from an individual person or animal can be substituted for the cell lines described, when testing for an individual's sensitivity to nongenotoxic carcinogens. Only reagents and antibodies would therefore be packaged in kits to test individual susceptibility.

Although the present invention has been described in relation to particular embodiments thereof, many other variations and modifications and other uses will become apparent to those skilled in the art. It is preferred, therefore, that the present invention be limited not by the specific disclosure herein, but only by the appended claims.

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WHAT IS CLAIMED IS:

1. A method for determining growth propensity for a tissue sample or cell line, said method comprising measuring a parameter that is indicative of concentration, in said sample or cell line, of at least one cyclin dependent kinase, and correlating said growth propensity to said measurement.

2. The method of claim 1, wherein said measurement is a direct measurement of the concentration of cyclin dependent kinase.

3. The method of claim 1, wherein said parameter is the concentration of $p34^{cdc2}$ or of a cyclin dependent kinase detectable at an apparent molecular weight between 32 and 34 kDa on a polyacrylamide gel.

4. The method of claim 1, wherein said parameter is the level of tyrosylphosphorylation of cyclin dependent kinase.

5. A diagnostic method for determining whether a tissue or cell sample has undergone transformation to a cancerous phenotype, said method comprising measuring a parameter indicative of concentration, in said tissue or cell sample, of at least one cyclin dependent kinase, and correlating said measurement to likelihood of transformation.

6. The method of claim 5, wherein said measurement is a direct measurement of the concentration of cyclin dependent kinase.

7. The method of claim 5, wherein said parameter is the concentration of $p34^{cdc2}$ or of a cyclin dependent kinase detectable at an apparent molecular weight between 32 and 34 kDa on a polyacrylamide gel.

8. The method of claim 5, wherein said parameter is the level of tyrosylphosphorylation of cyclin dependent kinase.

9. A diagnostic method for determining a likelihood that a tissue or cell sample will undergo transformation to a cancerous phenotype, said method comprising measuring a parameter that is indicative of concentration, in said tissue or cell sample, of at least one cyclin dependent kinase, and correlating said likelihood to said measurement.

10. The method of claim 9, wherein said measurement is a direct measurement of the concentration of cyclin dependent kinase.

11. The method of claim 9, wherein said parameter is the concentration of $p34^{cdc2}$ or of a cyclin dependent kinase detectable at an apparent molecular weight between 32 and 34 kDa on a polyacrylamide gel.

12. The method of claim 9, wherein said parameter is the level of tyrosylphosphorylation of cyclin dependent kinase.

13. A method of measuring carcinogenicity of a test substance comprising contacting said test substance with cells or tissue capable of expressing cyclin dependent

kinase and thereafter measuring a parameter indicative of concentration, in said cells or tissue, of at least one cyclin dependent kinase, and correlating said carcinogenicity with said measurement.

14. The method of claim 13, wherein said measurement is a direct measurement of the concentration of cyclin dependent kinase.

15. The method of claim 13, wherein said substance is contacted with an assay system selected from the group consisting of an animal, a cell culture, cell lines, or a panel of tissue.

16. The method of claim 13, wherein said test substance is selected from the group consisting of polychlorinated biphenyls, hormones and peroxisome proliferators.

17. The method of claim 13, wherein said parameter is the level of an mRNA coding for a cyclin dependent kinase.

18. The method of claim 13, wherein said test substance is nongenotoxic.

19. The method of claim 13, wherein said test substance is nonmutagenic.

20. The method of claim 13, wherein said parameter is the concentration of $p34^{cdc2}$.

21. The method of claim 13, wherein said parameter is the concentration of a cyclin dependent kinase detectable at an apparent molecular weight between 32 and 34 kDa on a polyacrylamide gel.

22. The method of claim 13, wherein said parameter is the level of tyrosylphosphorylation of cyclin dependent kinase.

24. The method of claim 13, wherein said measurement is performed on a cell lysate selected from the group consisting of 3T3 and BNL-CL.2.

25. A method of measuring effectiveness of a putative antineoplastic agent comprising the steps of:

(A) providing a sample of transformed cells;

(B) contacting said transformed cells with said putative antineoplastic agent;

(C) measuring a parameter indicative of concentration, in said cells, of at least one cyclin dependent kinase; and

(D) determining whether, or to what extent, said measurement indicates a decrease in cyclin dependent kinase following step (B).

26. The method of claim 25, wherein said parameter is measured both before and after step B, and said antineoplastic agent is evaluated by comparing the measurements taken before step (B) with measurements taken after step (B).

27. A kit for measuring cyclin dependent kinase concentration in human or animal cell lysates, said kit

including antibodies to an antigen whose concentration is indicative of cyclin dependent kinase concentration in said lysates.

28. The kit of claim 27, further including means for producing a standard curve from a standard having known cyclin dependent kinase content, or an historical standard curve.

29. The kit of claim 27, further comprising at least one inhibitor selected from the group consisting of a phosphate inhibitor and a protease inhibitor.

30. The kit of claim 27, wherein said antibodies are anti-cyclin dependent kinase antibodies.

31. The kit of claim 30, where an anti CDK antibody is selected from the group consisting of anti-C-terminis cdc2-polyclonal antibodies and anti-PSTAIR antibodies.

32. The kit of claim 27, further comprising:

(A) a lysate buffer;

(B) a means for receiving lysate and antibodies such that cyclin dependent kinase in said lysate may bind said antibodies;

(C) labelled secondary antibodies; and

(D) a means of detecting and quantifying said secondary antibodies.

33. A kit for measuring cyclin dependent kinase concentration in human or animal tissue or extracts, said kit including antibodies to an antigen whose concentration

is indicative of cyclin dependent kinase concentration in said tissues or extracts.

34. The kit of claim 33, wherein said kit further includes an historical standard curve or a means for producing a standard curve from a standard having known cycle dependent kinase content.

35. The kit of claim 33, further comprising at least one inhibitor selected from the group consisting of a phosphate inhibitor and a protease inhibitor.

36. The kit of claim 33, wherein said antibodies are anti-cyclin dependent kinase antibodies.

37. The kit of claim 36, wherein an anti CDK antibody is selected from the group consisting of anti-C-terminis cdc2 polyclonal antibody and anti-PSTAIR antibodies.

38. The kit of claim 37, further comprising:

(A) a homogenization buffer;

(B) a means for receiving a homogenate of said tissue and for receiving said antibodies such that cyclin dependent kinase in said homogenate may bind said antibodies;

(C) labelled secondary antibodies; and

(D) a means of detecting and quantifying said secondary antibodies.

39. An immunohistochemistry kit for determining whether cells or tissues have undergone transformation to a cancerous phenotype or are likely to undergo such transformation, said kit comprising a slide for receiving

a thin tissue slice containing said cells and further comprising an antibody to an antigen whose concentration is indicative of concentration of at least one cyclin dependent kinase in said cell or tissue sample.

40. The kit of claim 39, wherein said slide also includes, as separate slices, positive and negative control tissue.

41. The kit of claim 39, wherein said kit further includes an historical standard curve or means for producing a standard curve from a standard having known CDK content.

42. The kit of claim 39, further comprising at least one inhibitor selected from the group consisting of a phosphate inhibitor and a protease inhibitor.

43. The kit of claim 39, wherein the kit includes anti CDK antibody.

44. A method for determining efficacy of a regimen for reducing or enhancing cell growth, said method comprising the steps of measuring a parameter indicative of concentration levels of at least one cyclin dependent kinase following treatment of those cells with said regimen and correlating cyclin dependent kinase concentration with said efficacy.

45. The method of claim 44, wherein cyclin dependent kinase concentration is measured before and after beginning said regimen and said determination comprises comparing the measurement taken before with the measurement taken after.

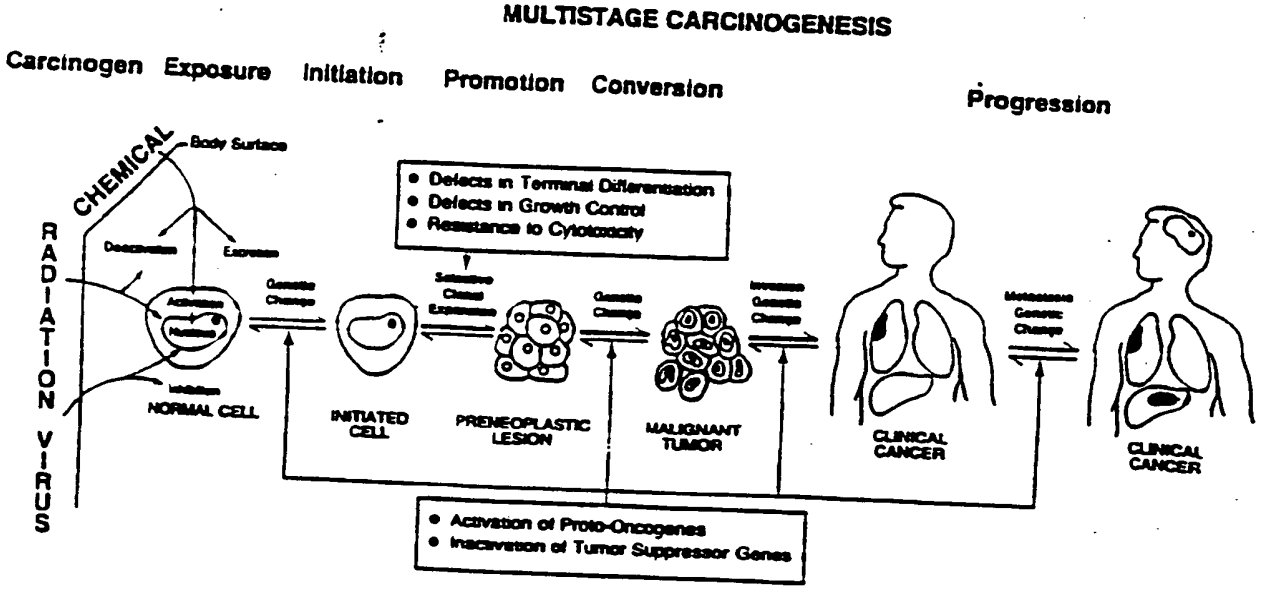
MULTISTAGE CARCINOGENESIS
Carcinogen Exposure
Initiation
Promotion
Conversion
Progression
CHEMICAL
RADIATION
VIRUS
Body Surface
Deactivation
Excretion
Activation
Mutation
Inhibition
NORMAL CELL
Genetic Change
INITIATED CELL
Selective Clonal Expansion
PREMEOPLASTIC LESION
MALIGNANT TUMOR
Invasion
CLINICAL CANCER
Metastasis
CLINICAL CANCER
• Defects in Terminal Differentiation
• Defects in Growth Control
• Resistance to Cytotoxicity
• Activation of Proto-Oncogenes
• Inactivation of Tumor Suppressor Genes

FIG. 1

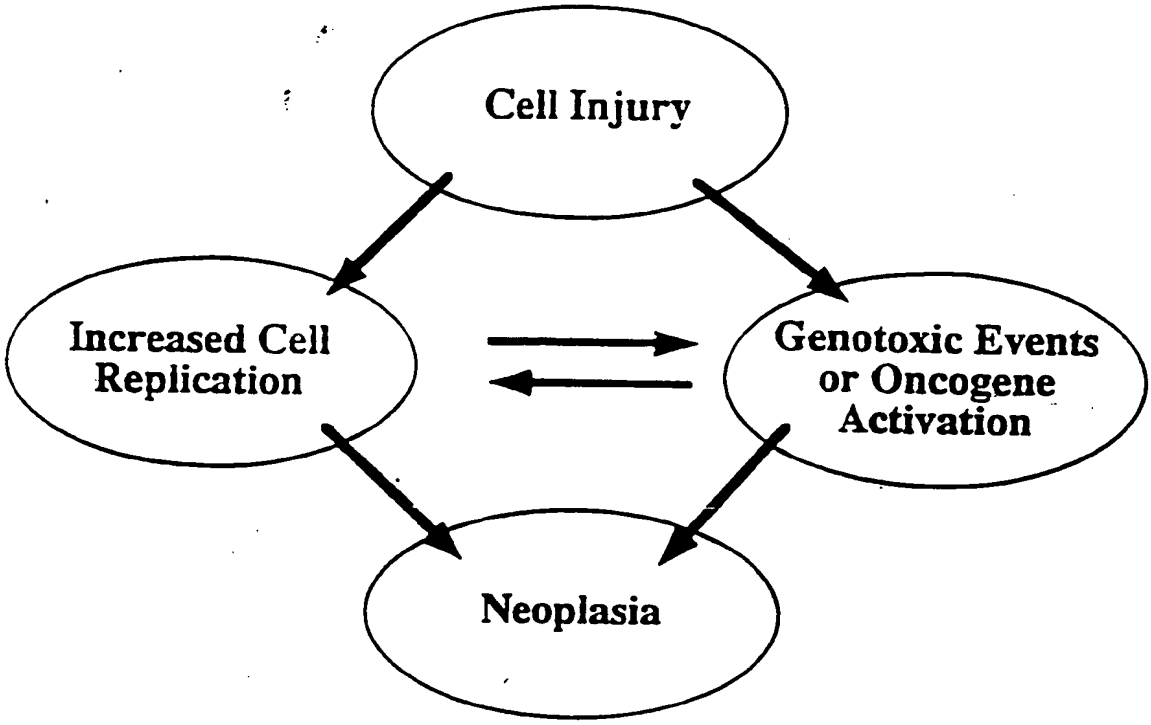
Cell Injury
Increased Cell Replication
Genotoxic Events or Oncogene Activation
Neoplasia

FIG. 2

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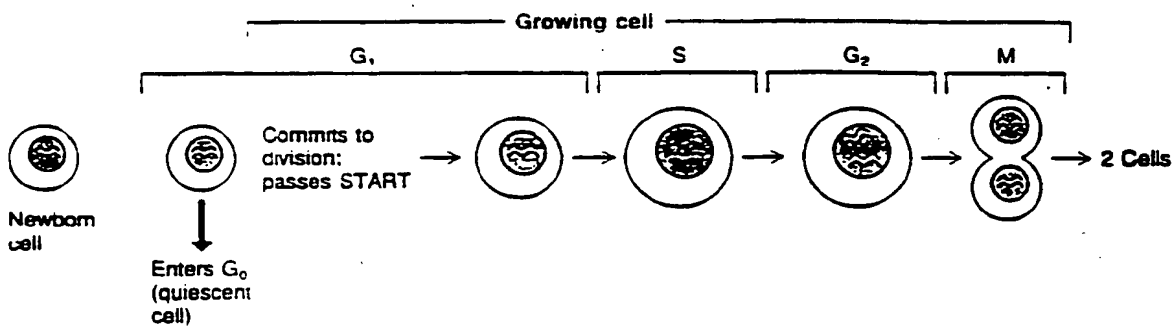

Growing cell
G1
S
G2
M
Newborn cell
Commits to division: passes START
2 Cells
Enters Go (quiescent cell)

FIG. 3

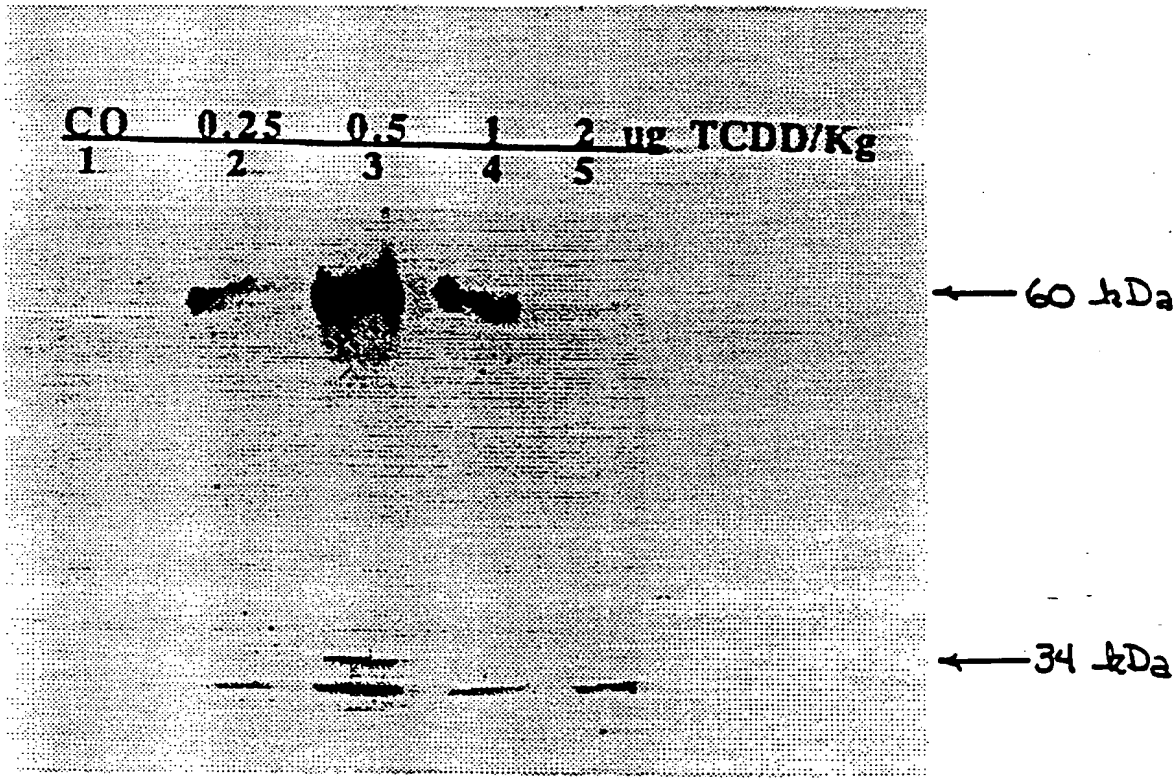
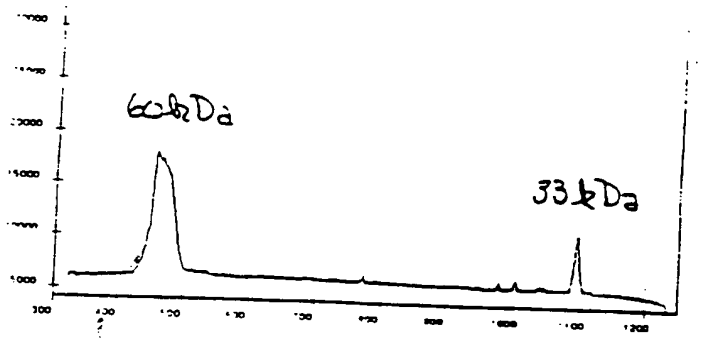
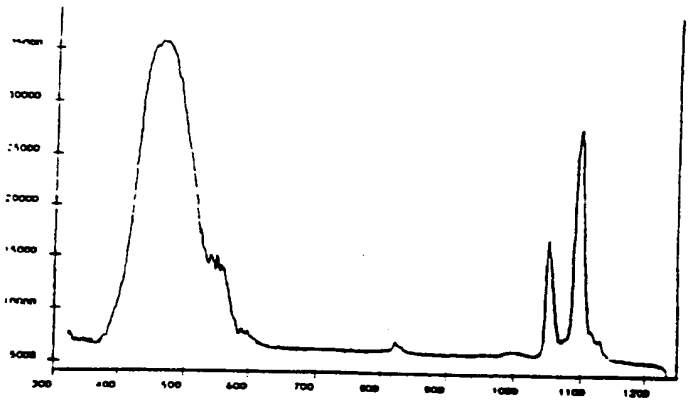
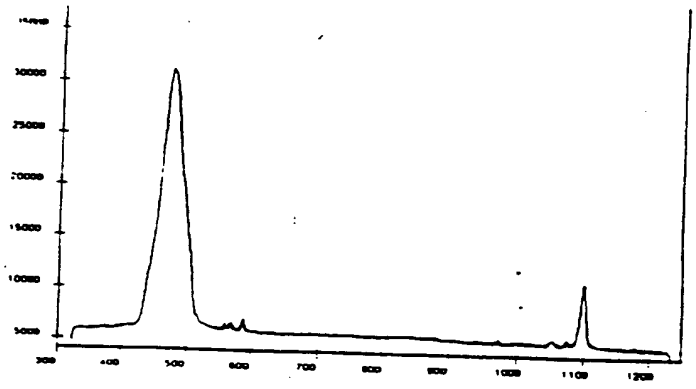


FIG. 4

34cdc 2







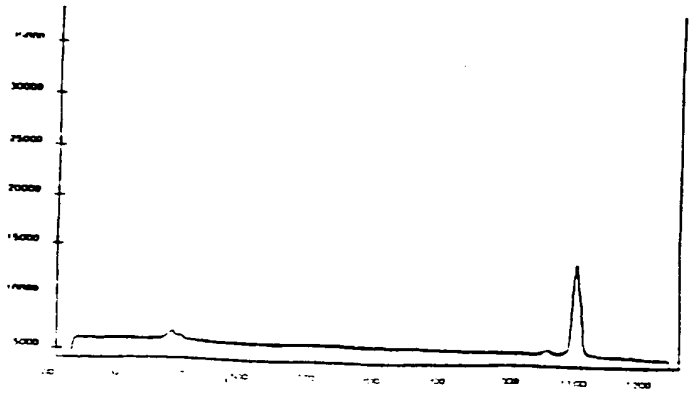


FIG. 5

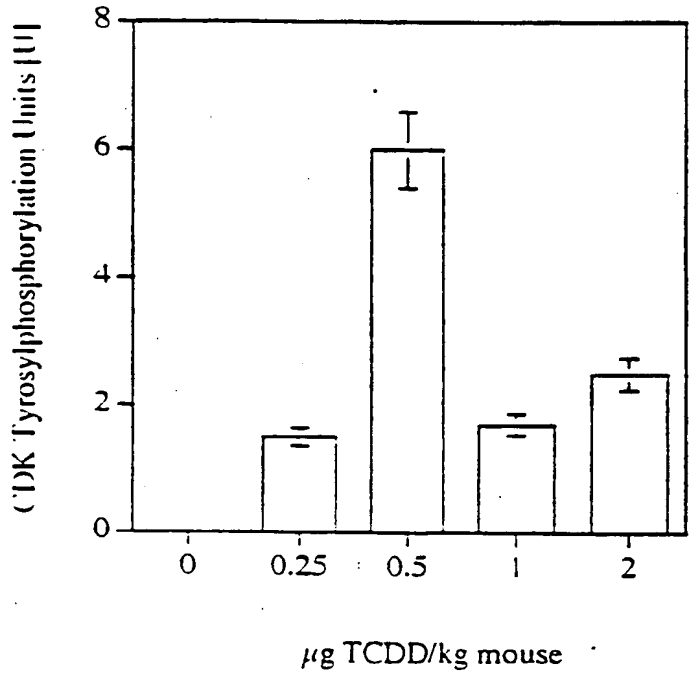

EDK Tyrosylphosphorylation Units [U]
8
6
4
2
0
0
0.25
0.5
1
2
μg TCDD/kg mouse

FIG. 6

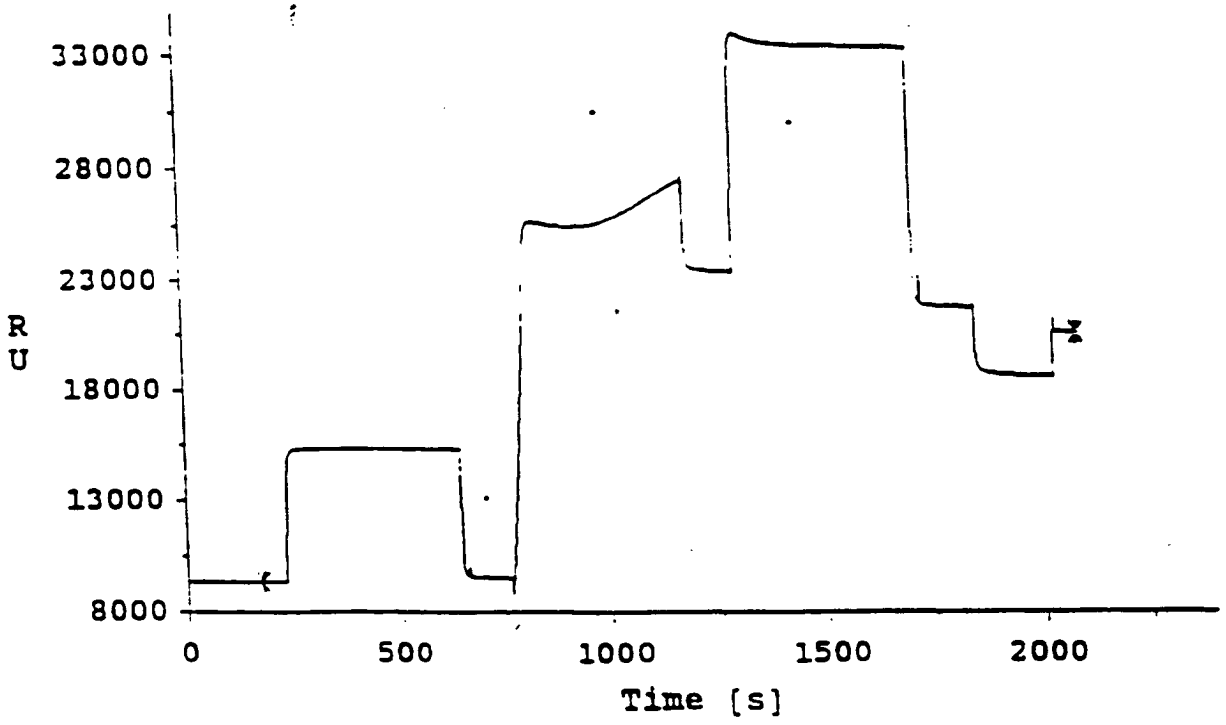

33000
28000
23000
R
U
18000
13000
8000
0
500
1000
1500
2000
Time [s]

FIG. 7

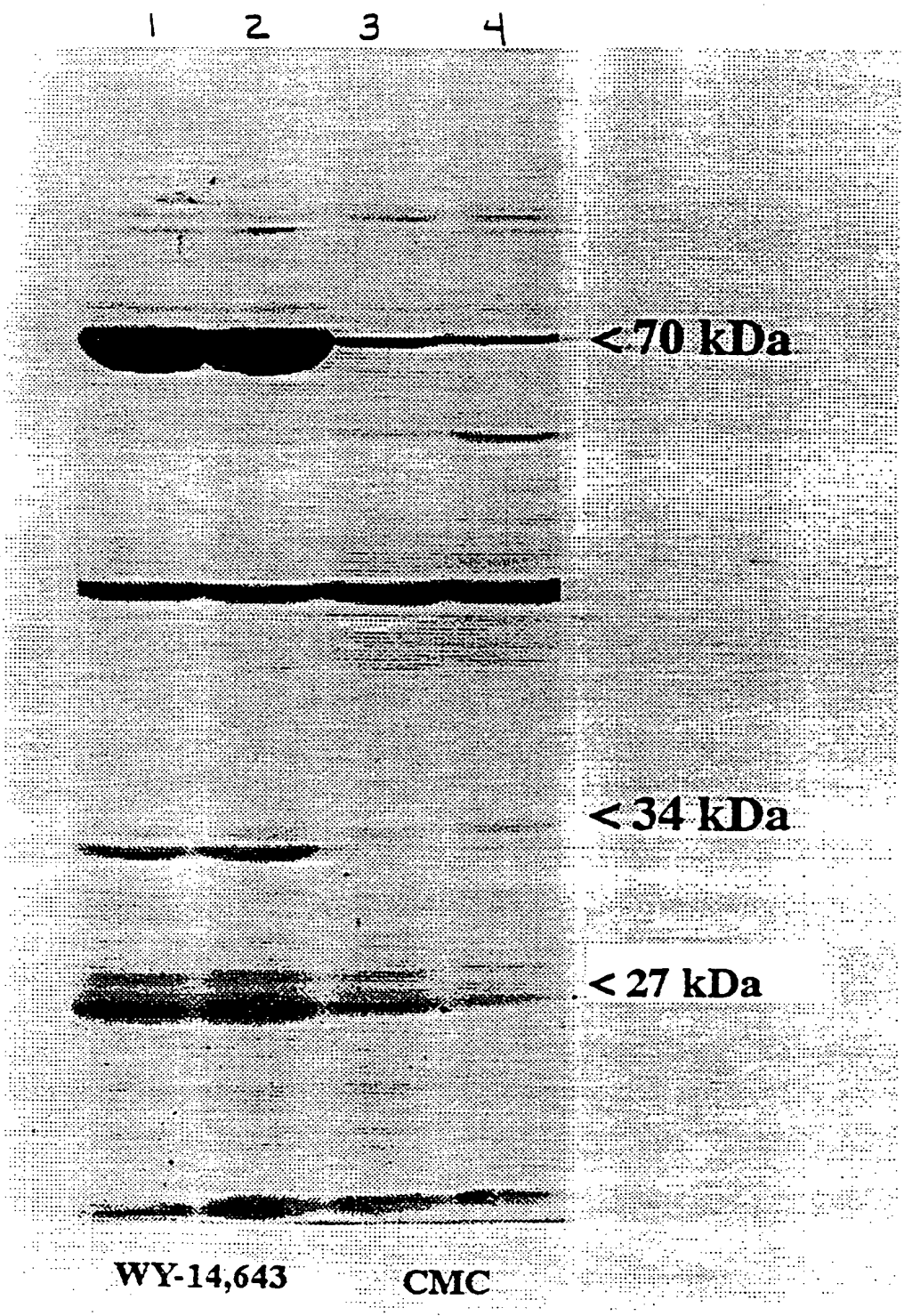
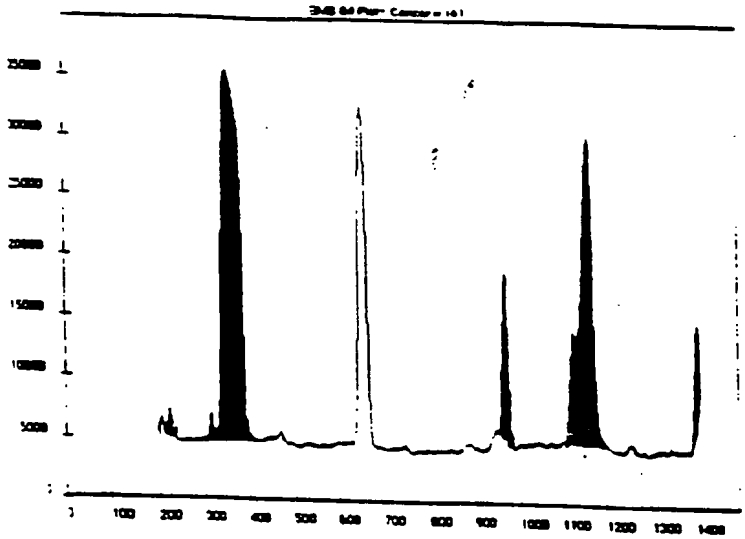
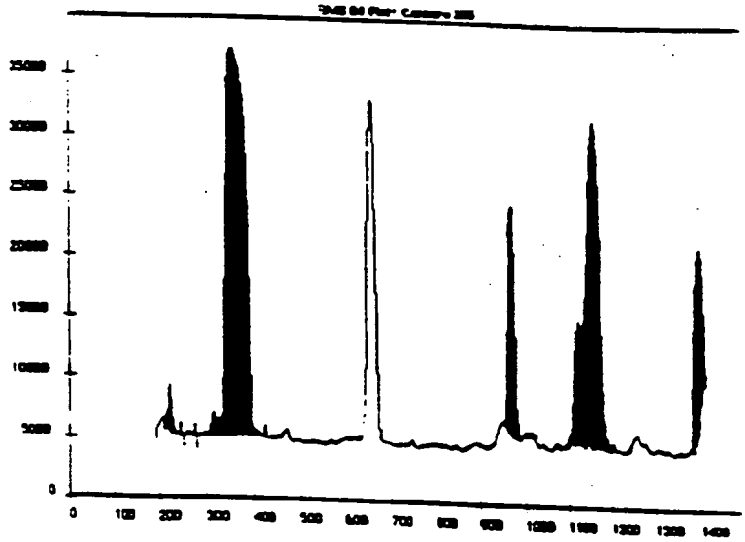


FIG. 8

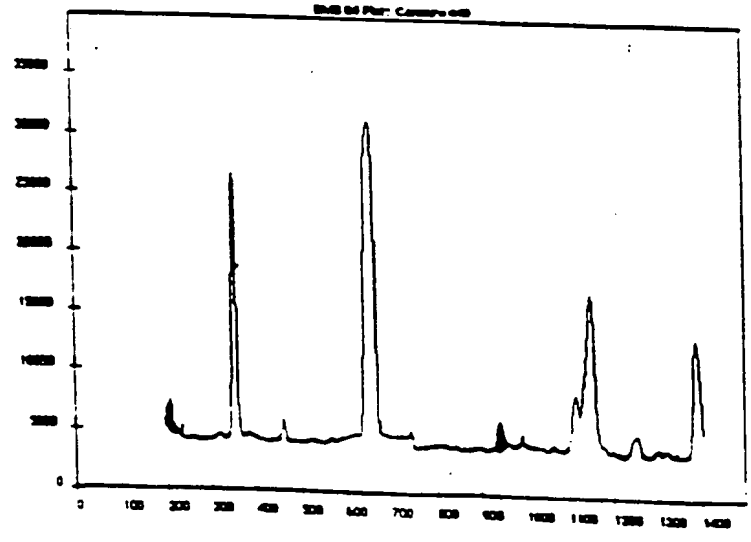
A



B



C



D

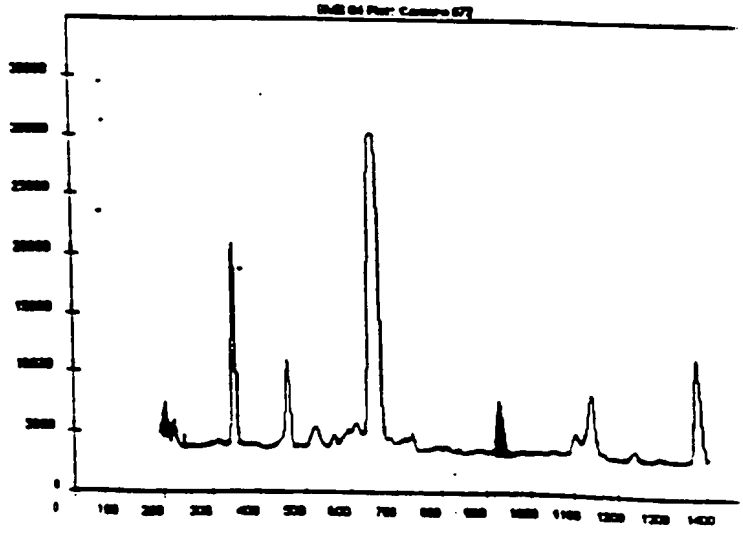


FIG 9

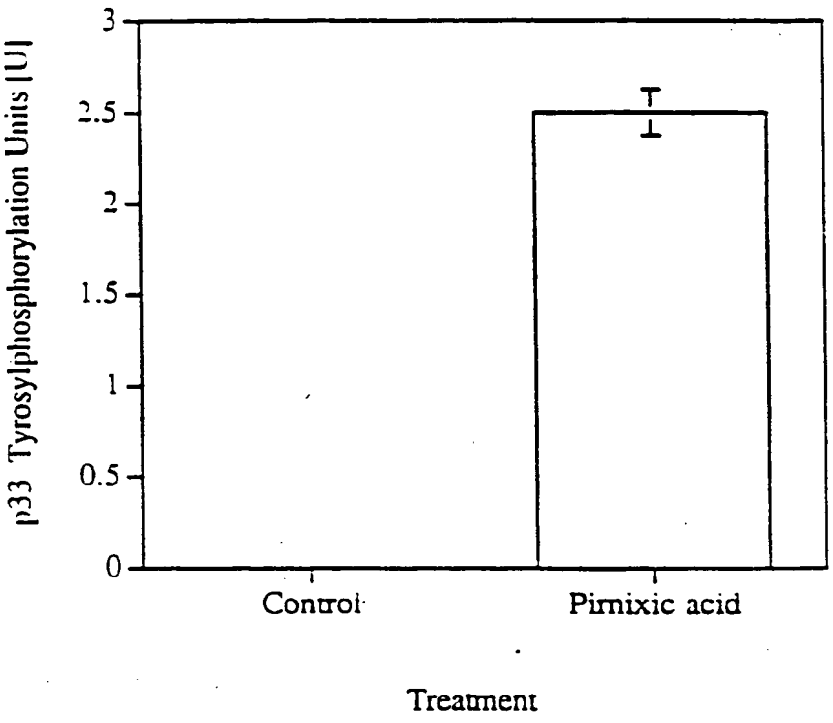

p33 Tyrosylphosphorylation Units [U]
3
2.5
2
1.5
1
0.5
0
Control
Pirnixic acid
Treatment

FIG. 10

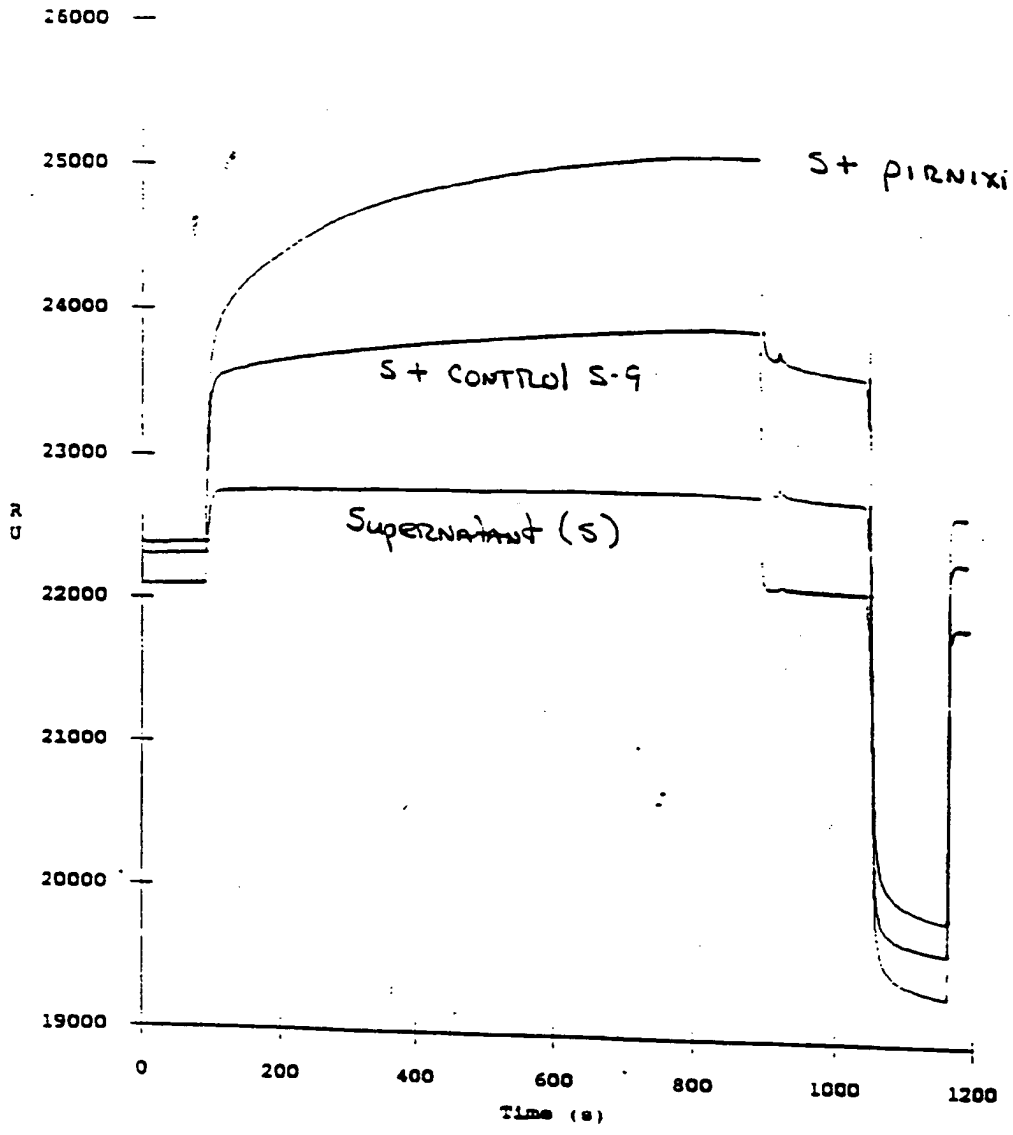

26000
25000
24000
23000
22000
21000
20000
19000
RU
0
200
400
600
800
1000
1200
Time (s)
S + PIRNIXIC Acid S-9
S + CONTROL S-9
Supernatant (S)

FIG 11

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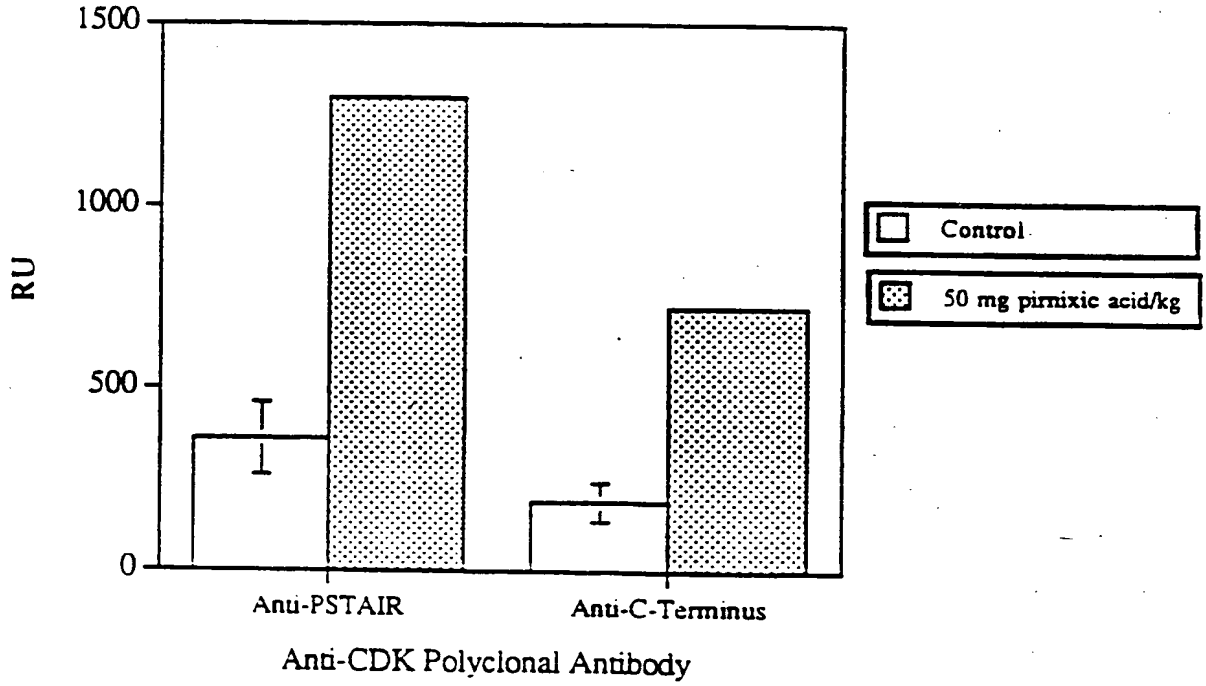

1500
1000
500
0
RU
Control
50 mg pirmixic acid/kg
Anti-PSTAIR
Anti-C-Terminus
Anti-CDK Polyclonal Antibody

FIG. 12

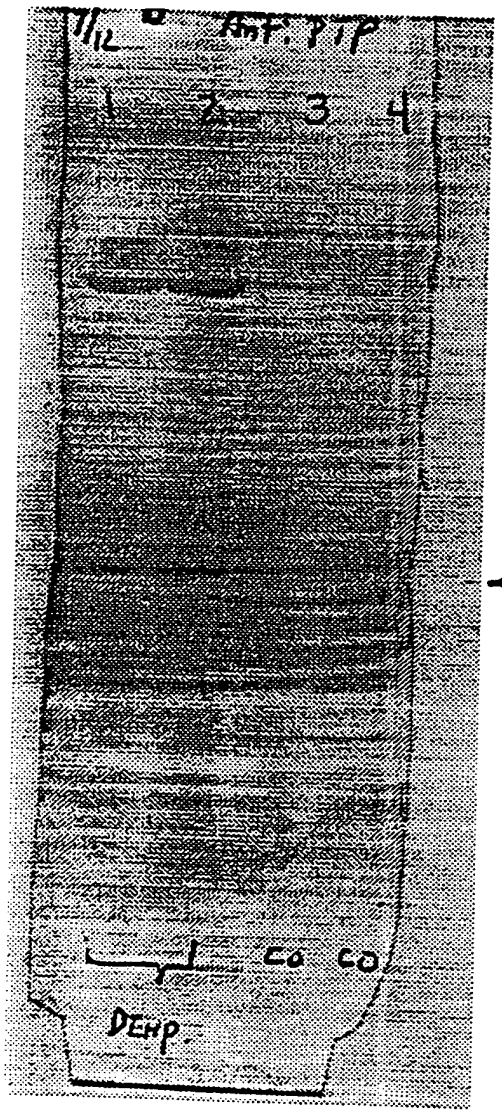
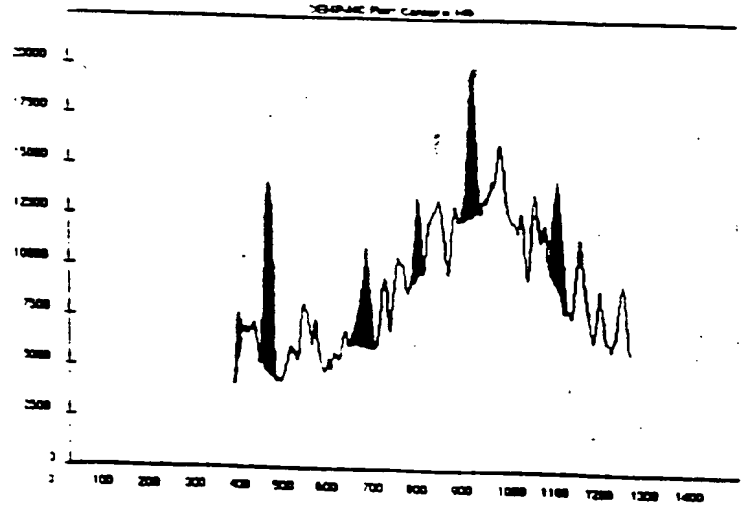
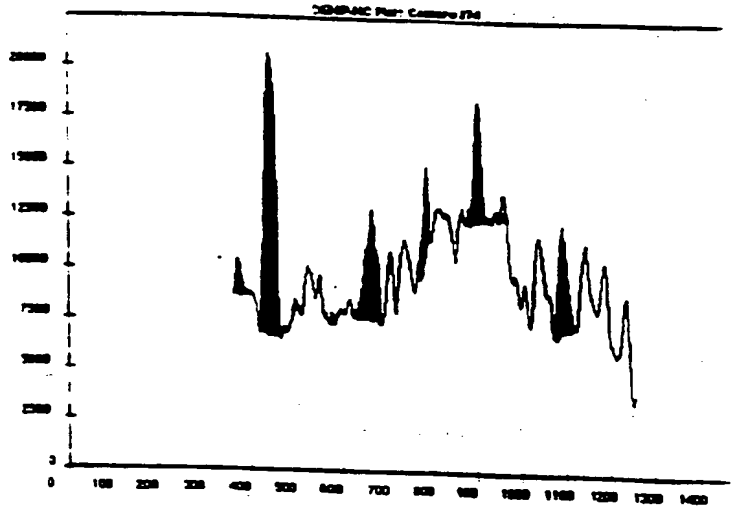


FIG. 13

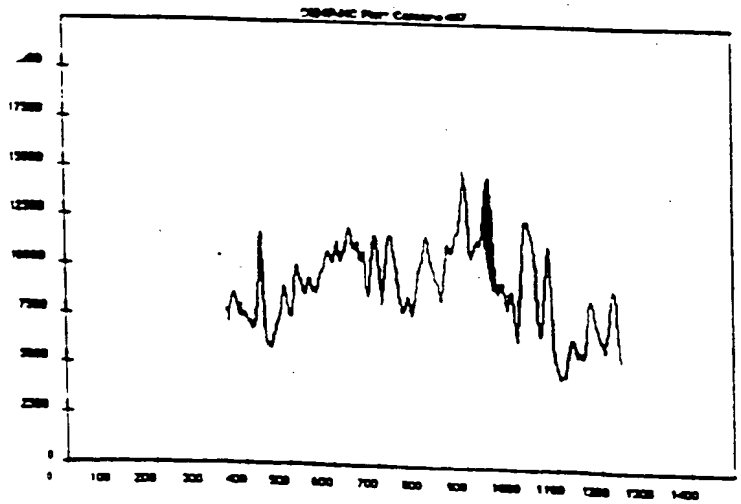
A



B



C



D

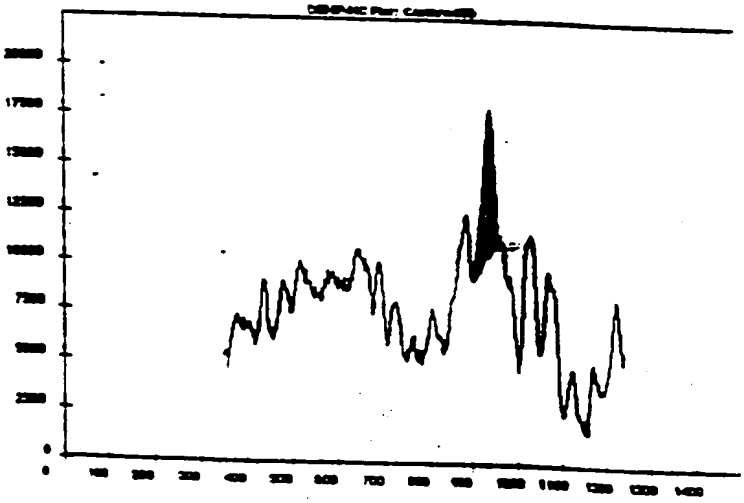


FIG. 14

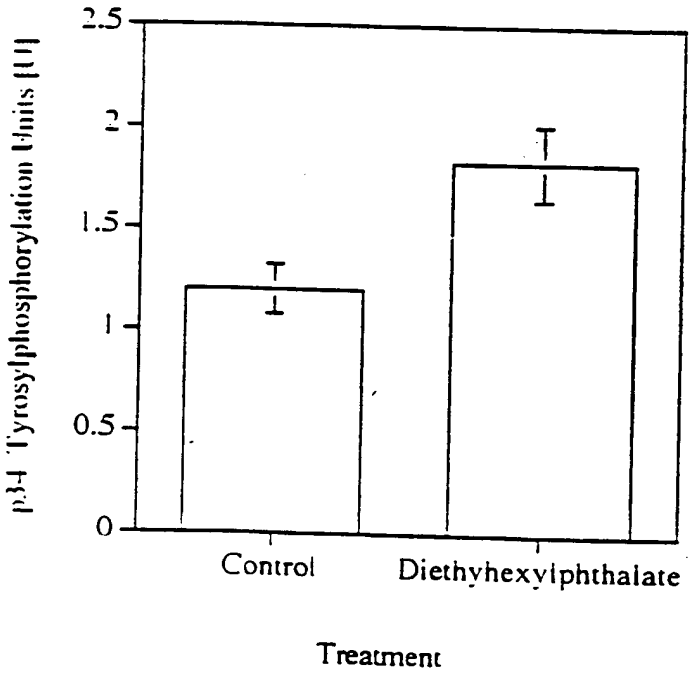

p34 Tyrosylphosphorylation Units [U]
2.5
2
1.5
1
0.5
0
Control
Diethyhexylphthalate
Treatment

FIG. 15

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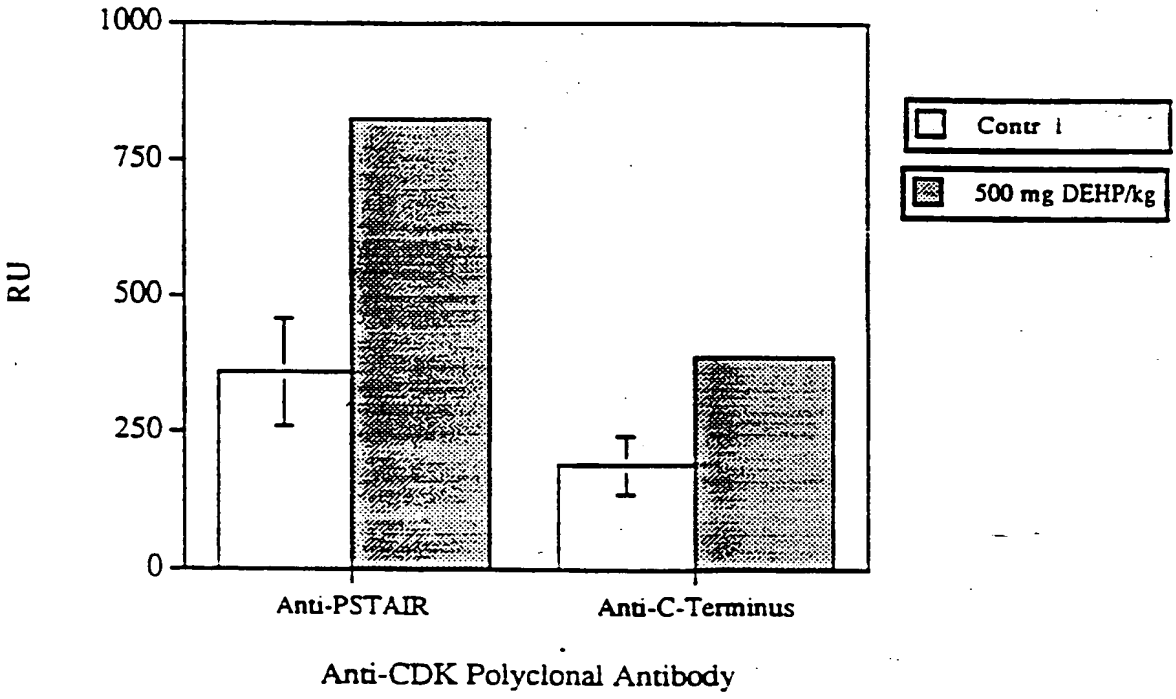

1000
750
500
250
0
RU
Anti-PSTAIR
Anti-C-Terminus
Anti-CDK Polyclonal Antibody
Contr l
500 mg DEHP/kg

FIG 16

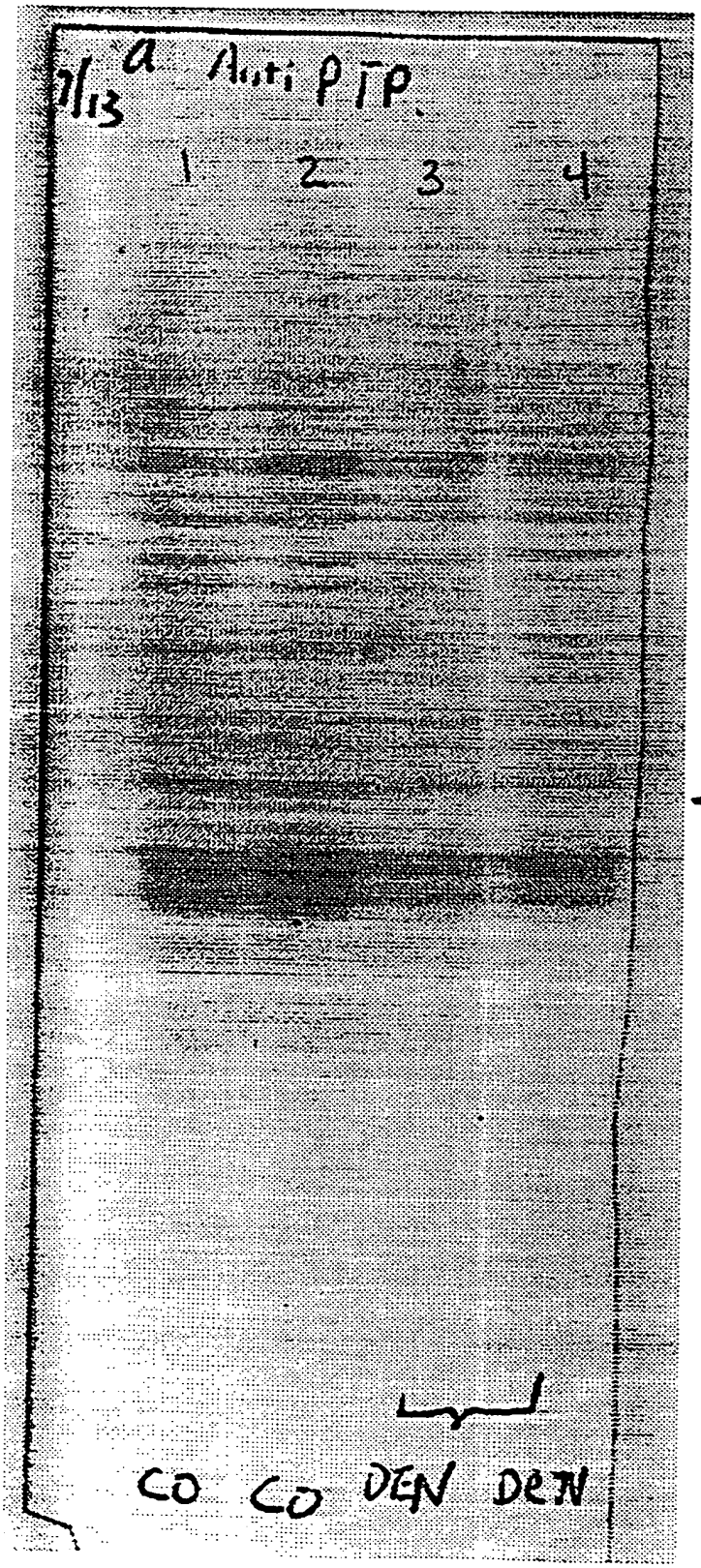
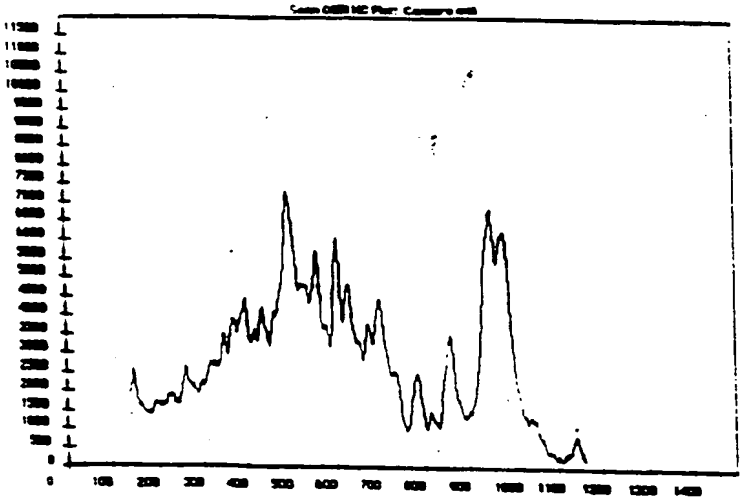
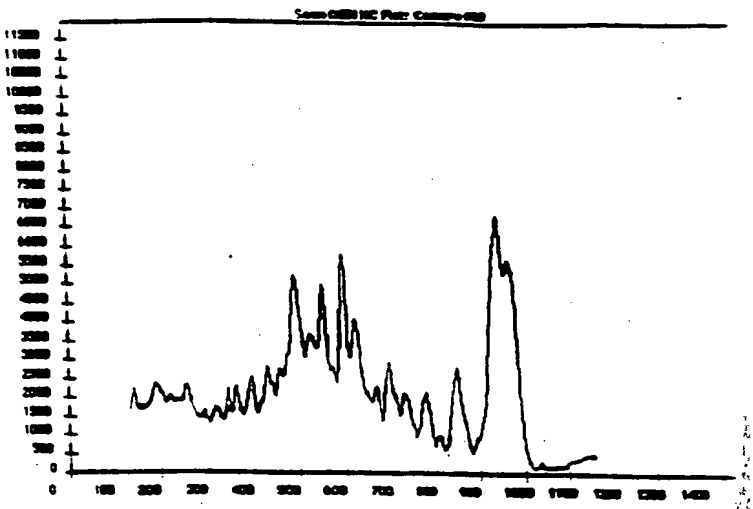


FIG. 17

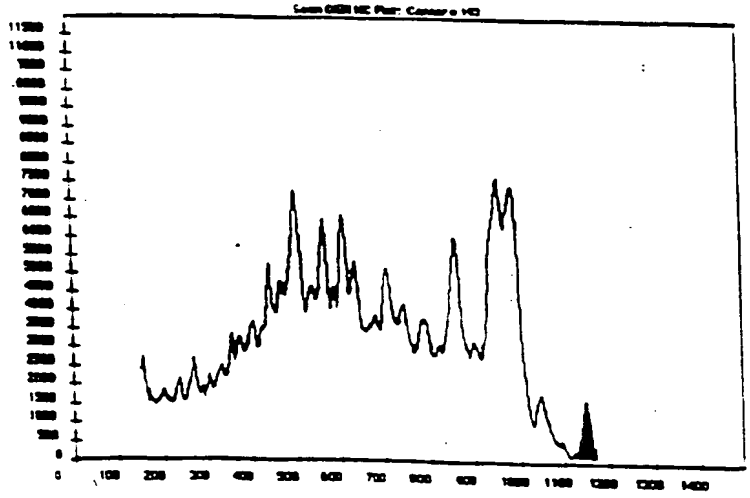
A



B



C



D

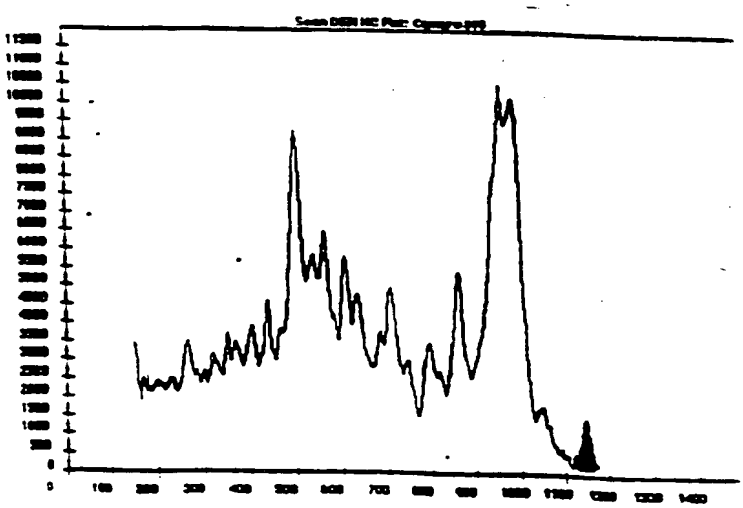


FIG. 18

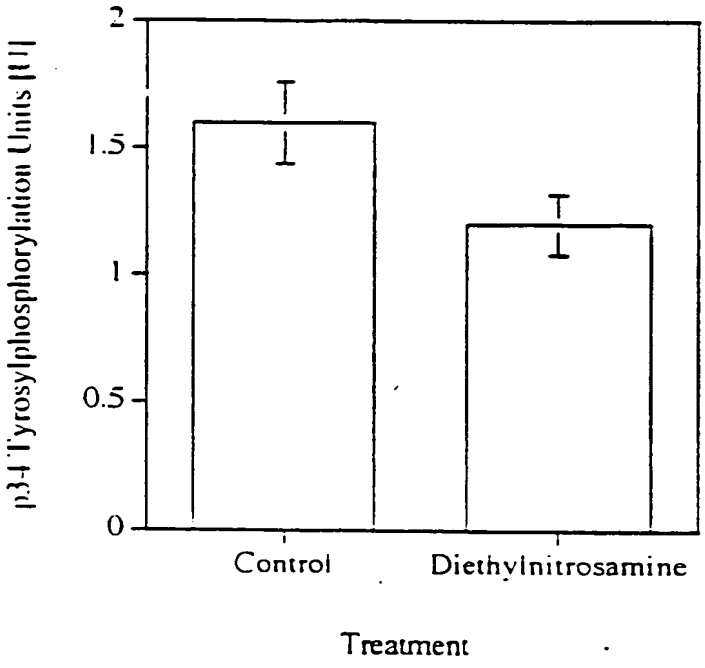

p34 Tyrosylphosphorylation Units [1]
2
1.5
1
0.5
0
Control
Diethylnitrosamine
Treatment

FIG. 19

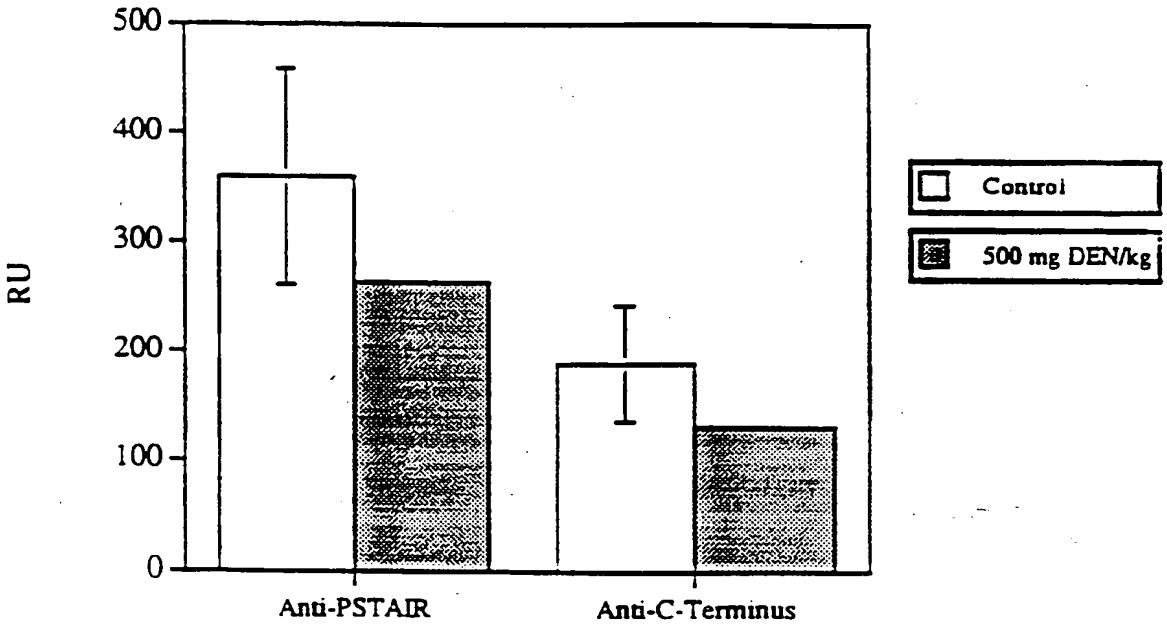
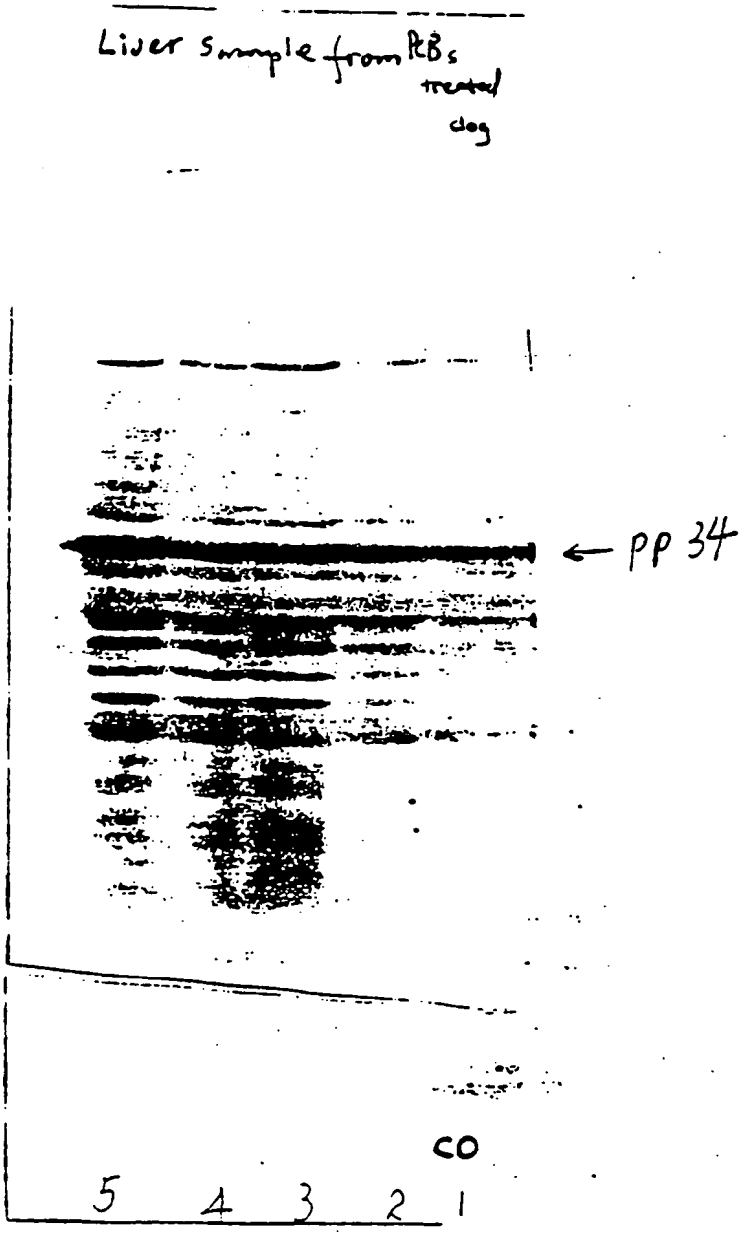

500
400
300
200
100
0
RU
Anti-PSTAIR
Anti-C-Terminus
Anti-CDK Polyclonal Antibody
Control
500 mg DEN/kg

FIG. 20

FIG. 21



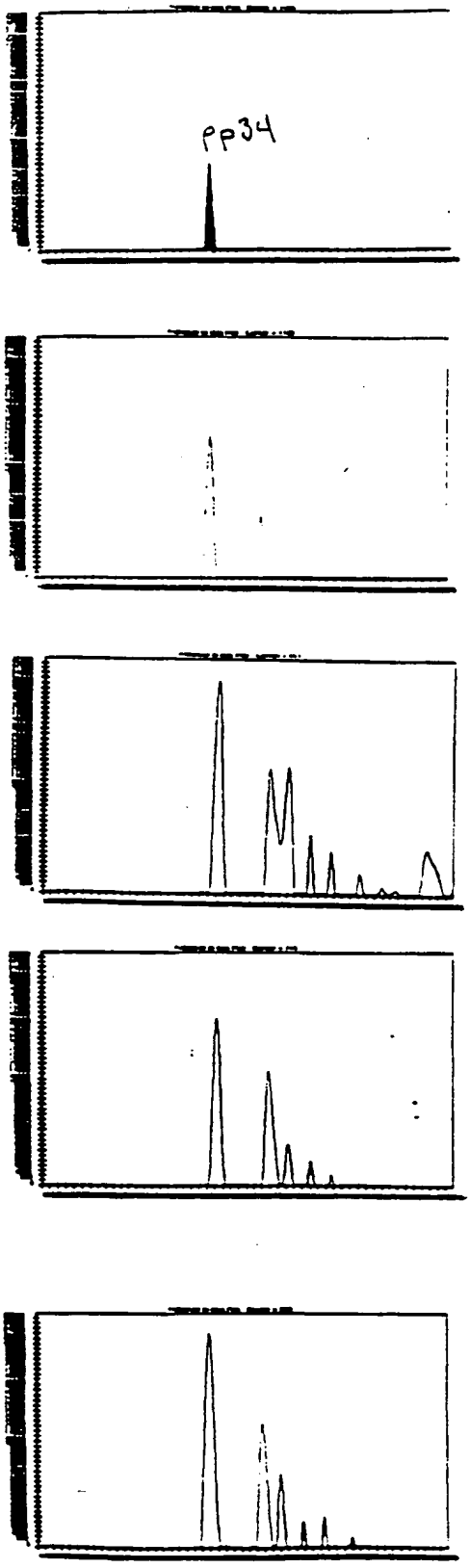


FIG. 22

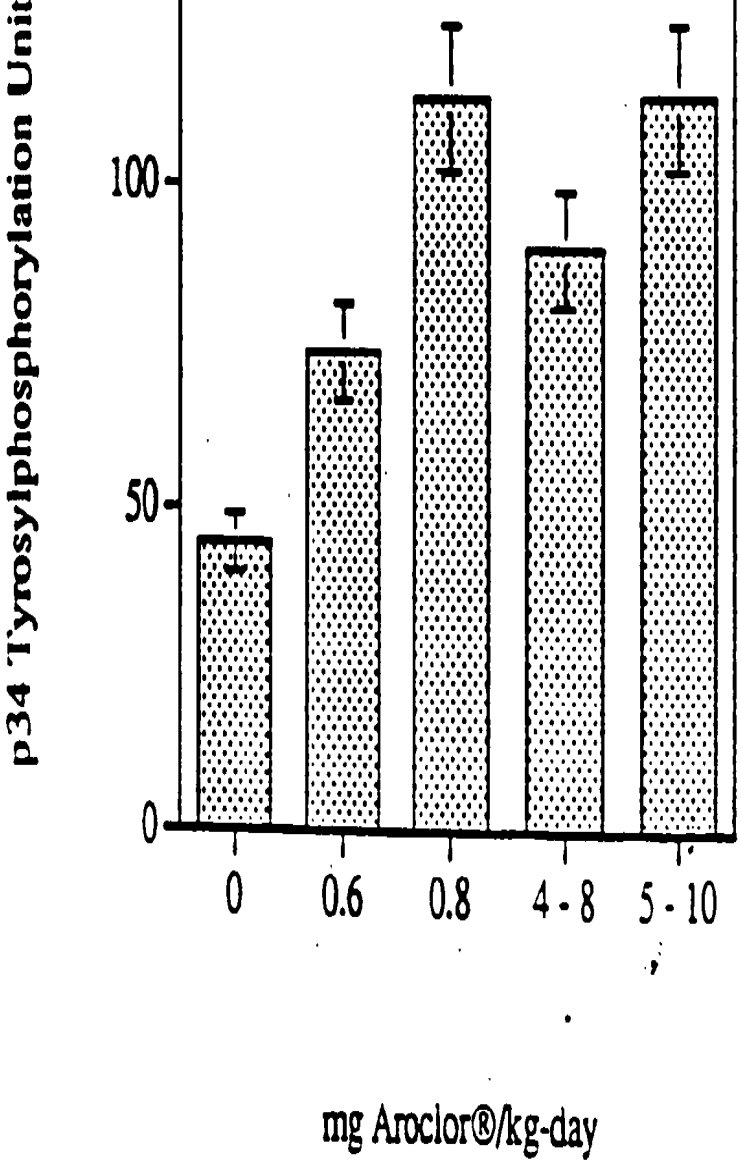

p34 Tyrosylphosphorylation Units
100
50
0
0
0.6
0.8
4 - 8
5 - 10
mg Aroclor®/kg-day

FIG. 23

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FIG. 24

3T3 cell line

with Anti-phosphorylation tyrosine Antibody

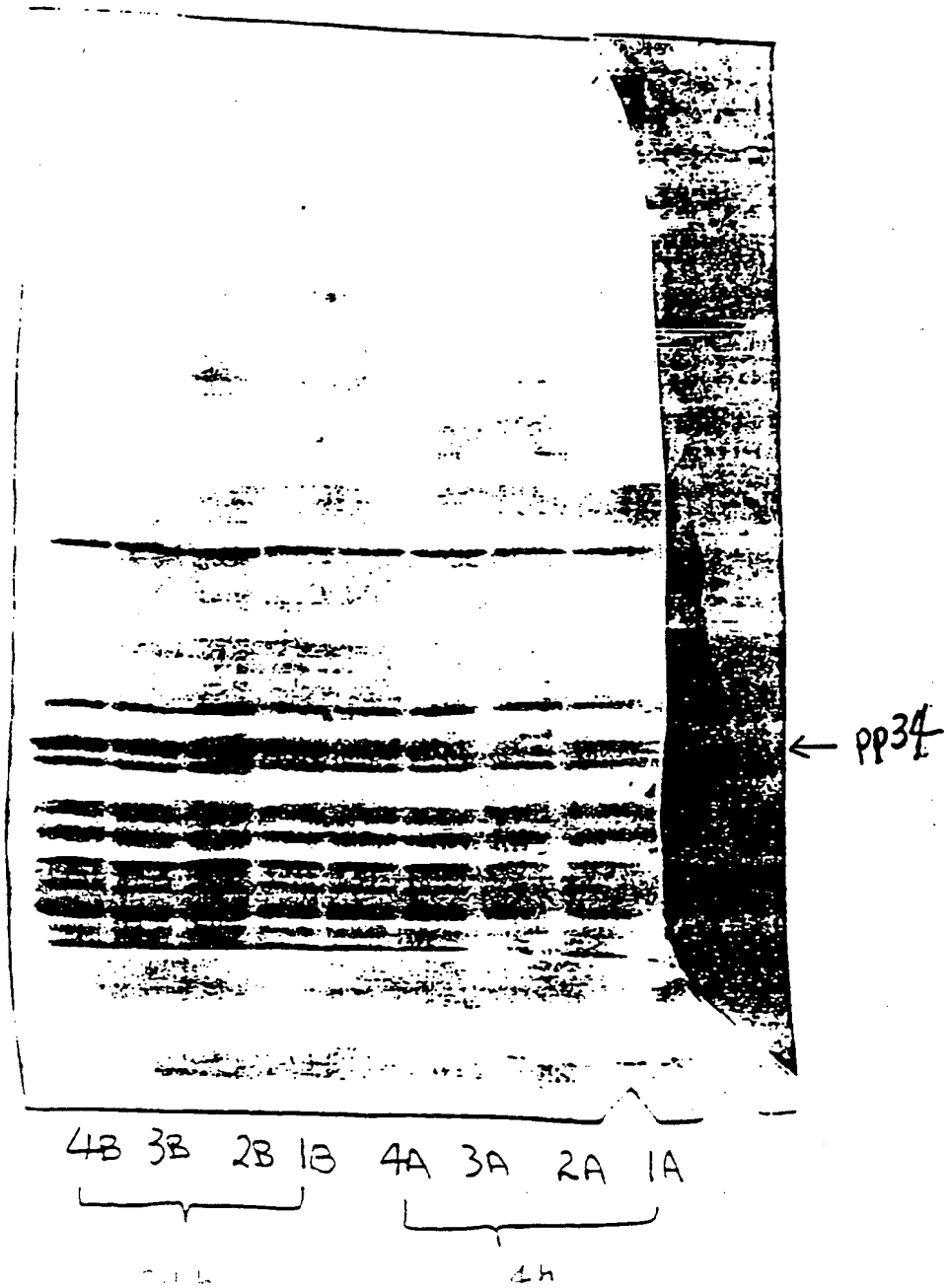
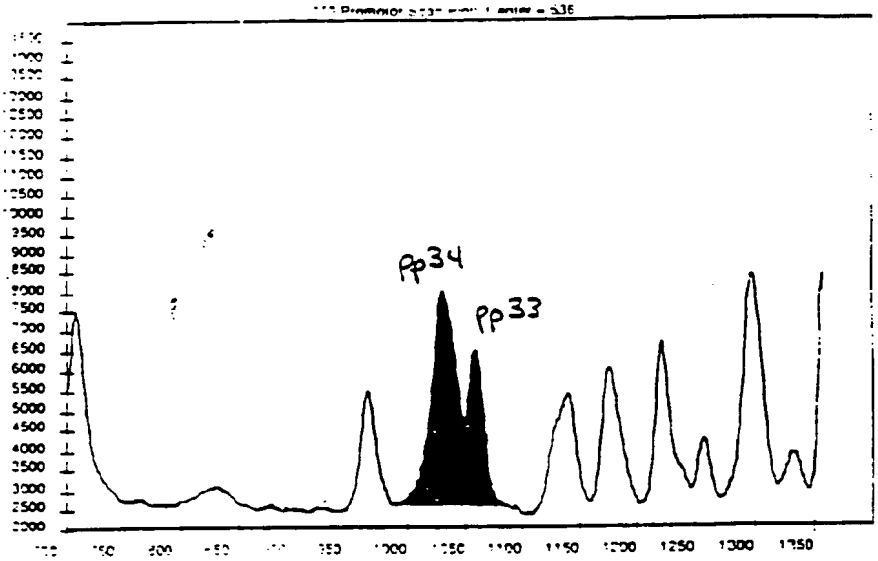
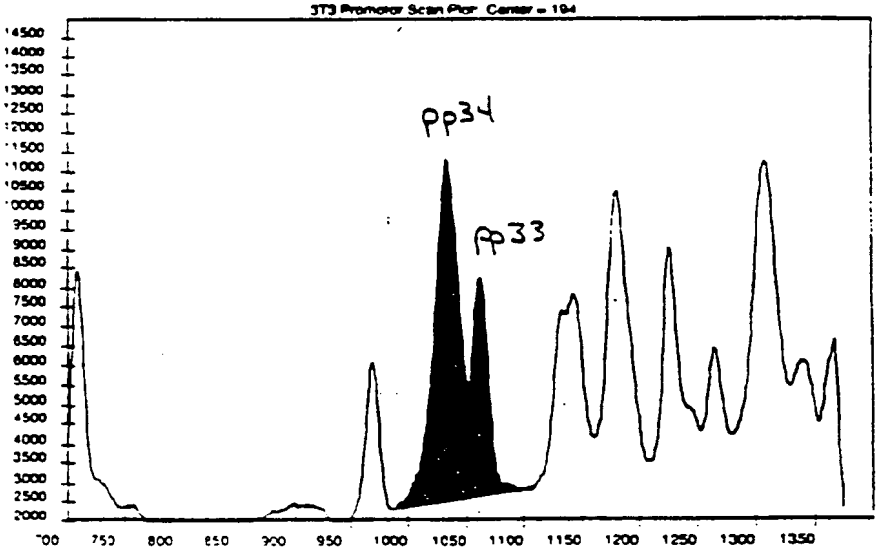


FIG. 25


pp34
pp33
700
750
800
850
900
950
1000
1050
1100
1150
1200
1250
1300
1350


3T3 Promoter Scan Plot Center = 194
pp34
pp33
700
750
800
850
900
950
1000
1050
1100
1150
1200
1250
1300
1350

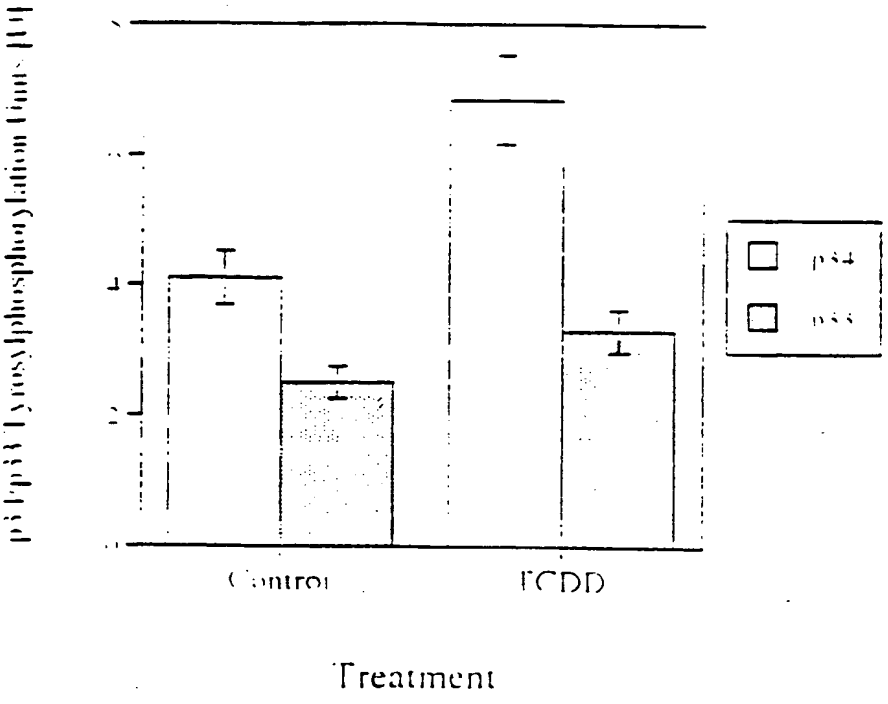
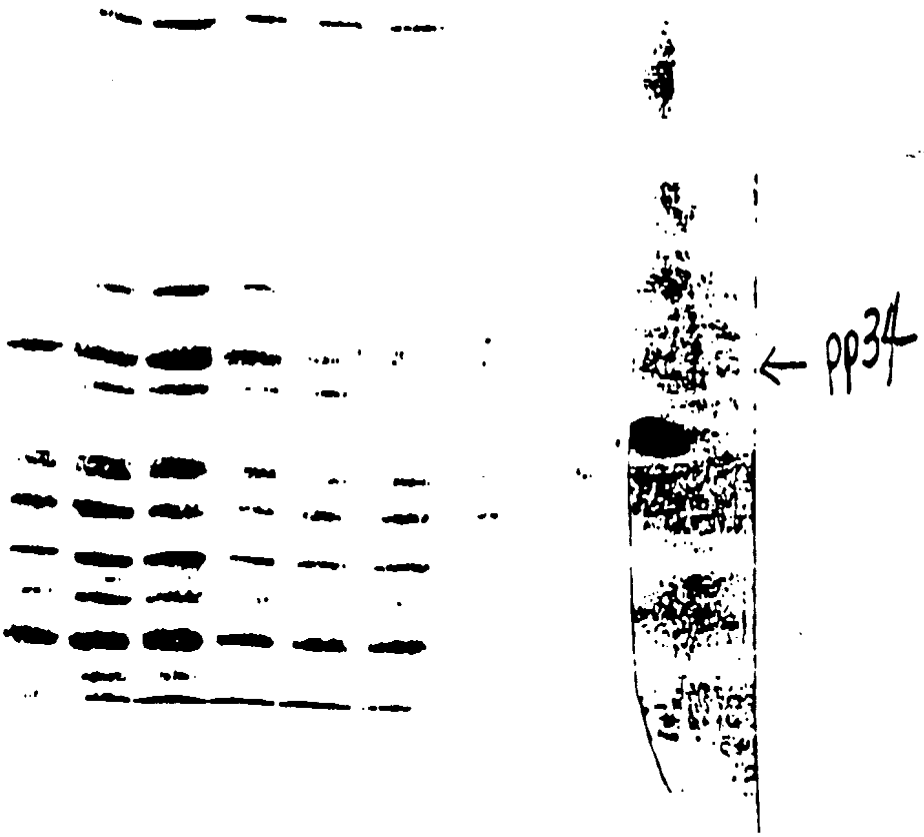
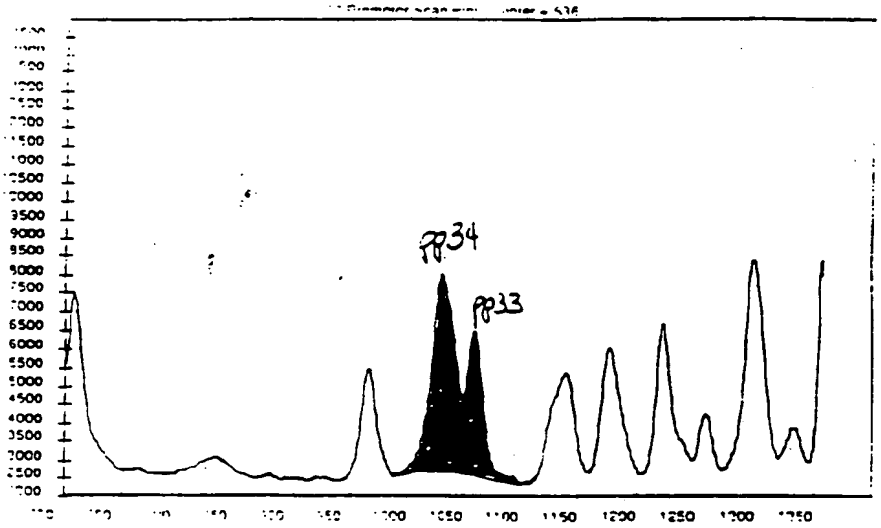


FIG. 26


← pp34
4B 3B 2B 1B 4A 3A 2A 1A



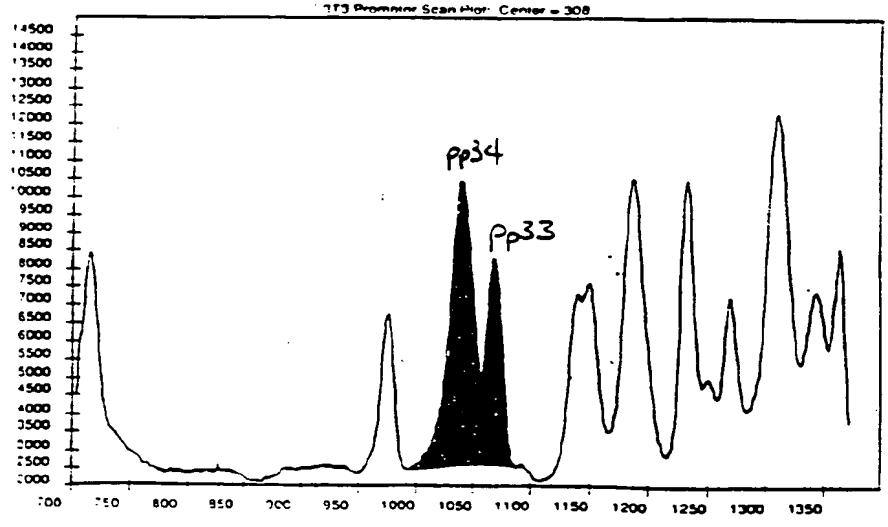


FIG. 28

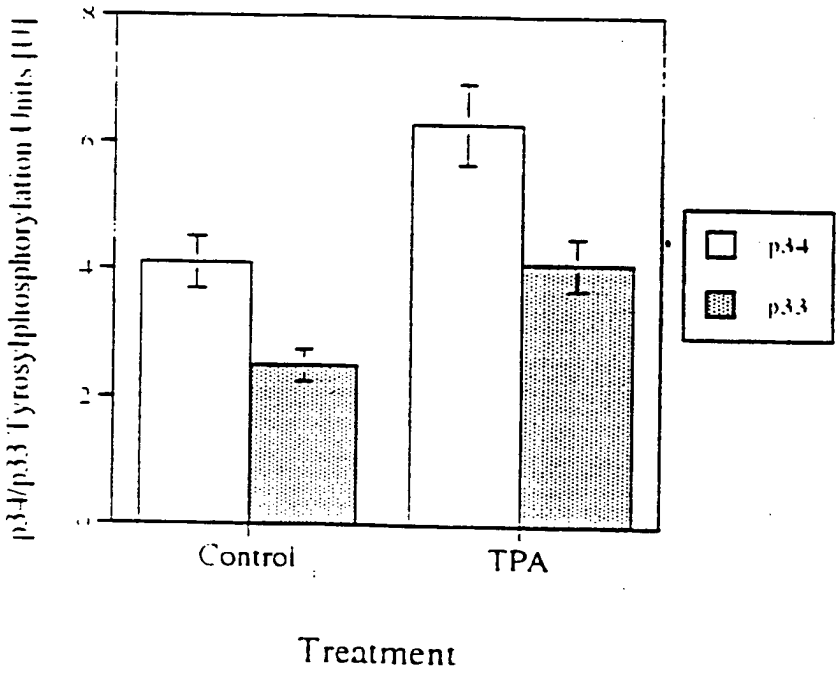
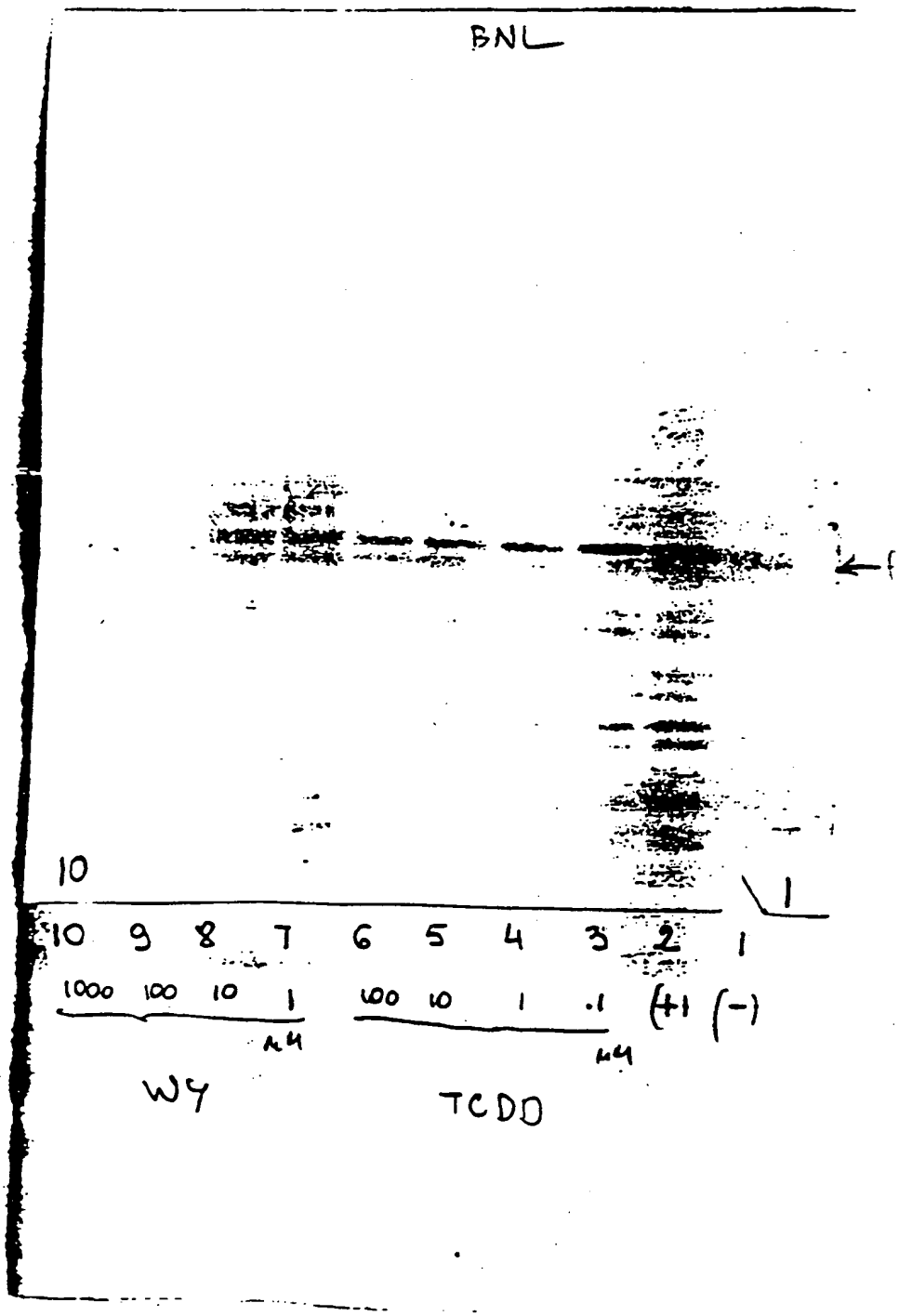

p34/p33 Tyrosylphosphorylation Units [II]
Control
TPA
Treatment
p34
p33

FIG. 29


BNL
10
1
10 9 8 7 6 5 4 3 2 1
1000 100 10 1 100 10 1 .1 (+) (-)
nM nM
WY
TCDD

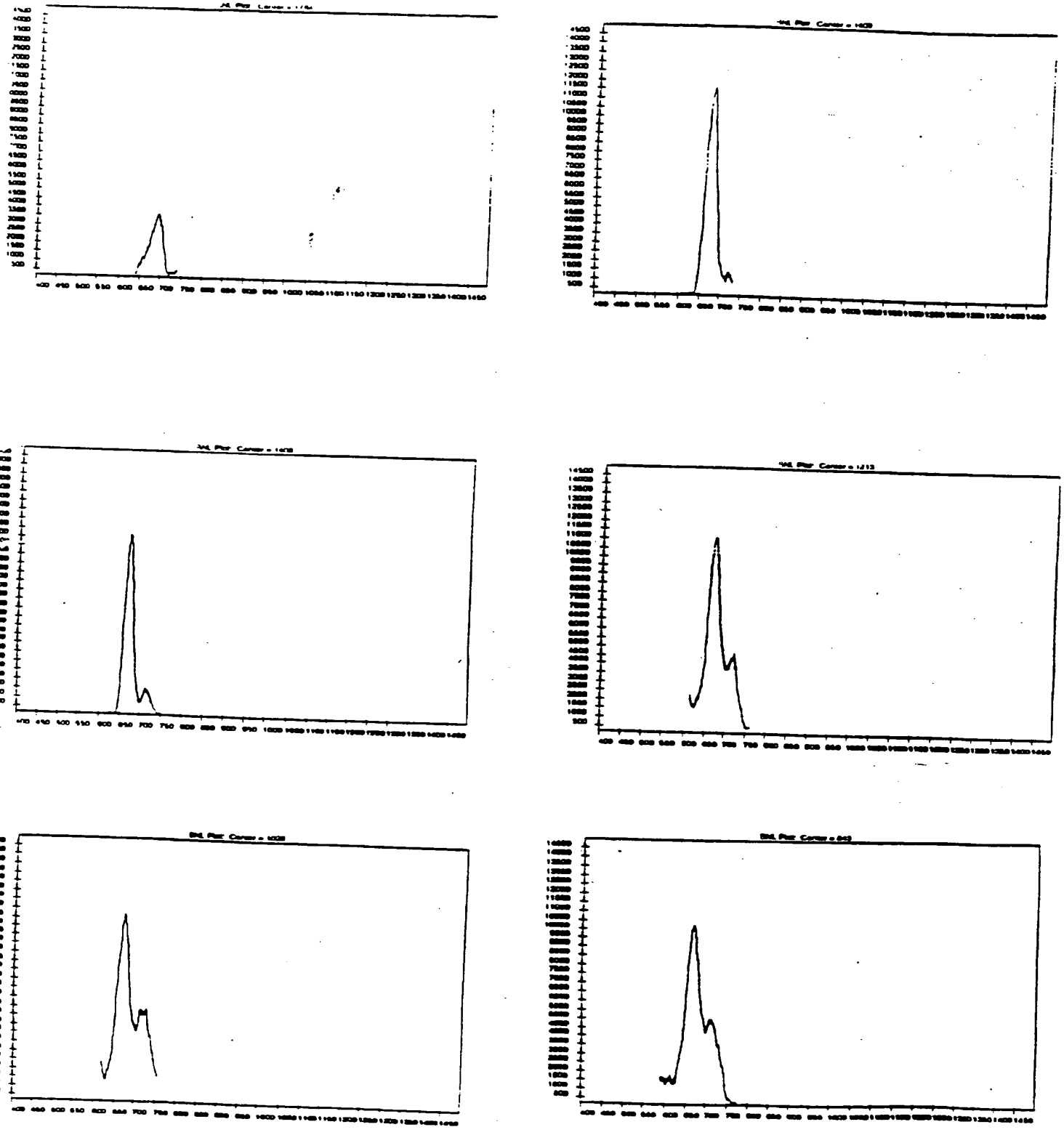


FIG. 31

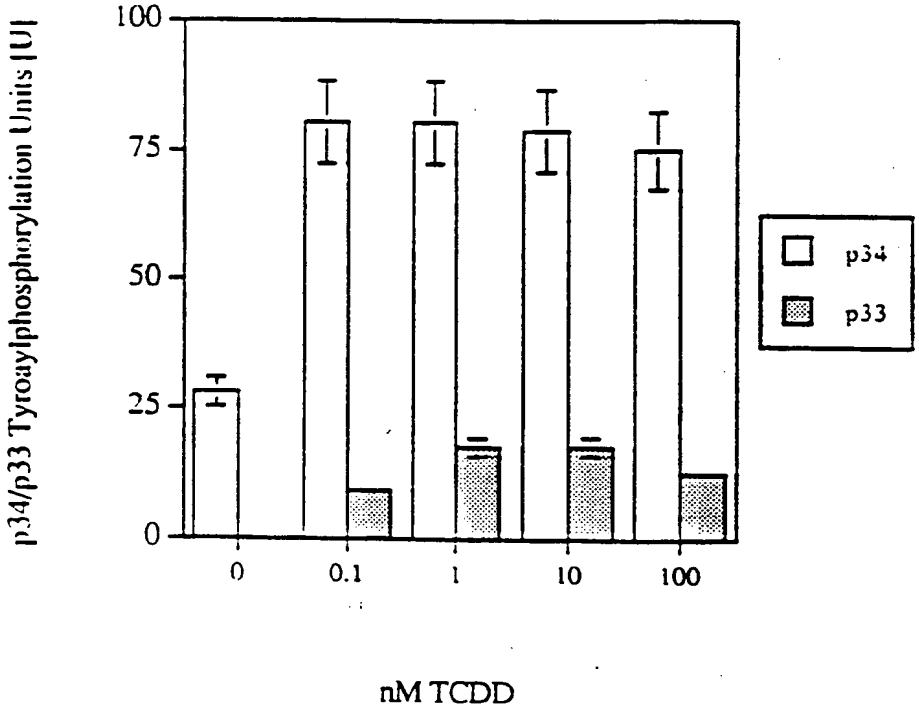

p34/p33 Tyroaylphosphorylation Units [U]
100
75
50
25
0
0
0.1
1
10
100
nM TCDD
p34
p33

FIG. 32

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BNL

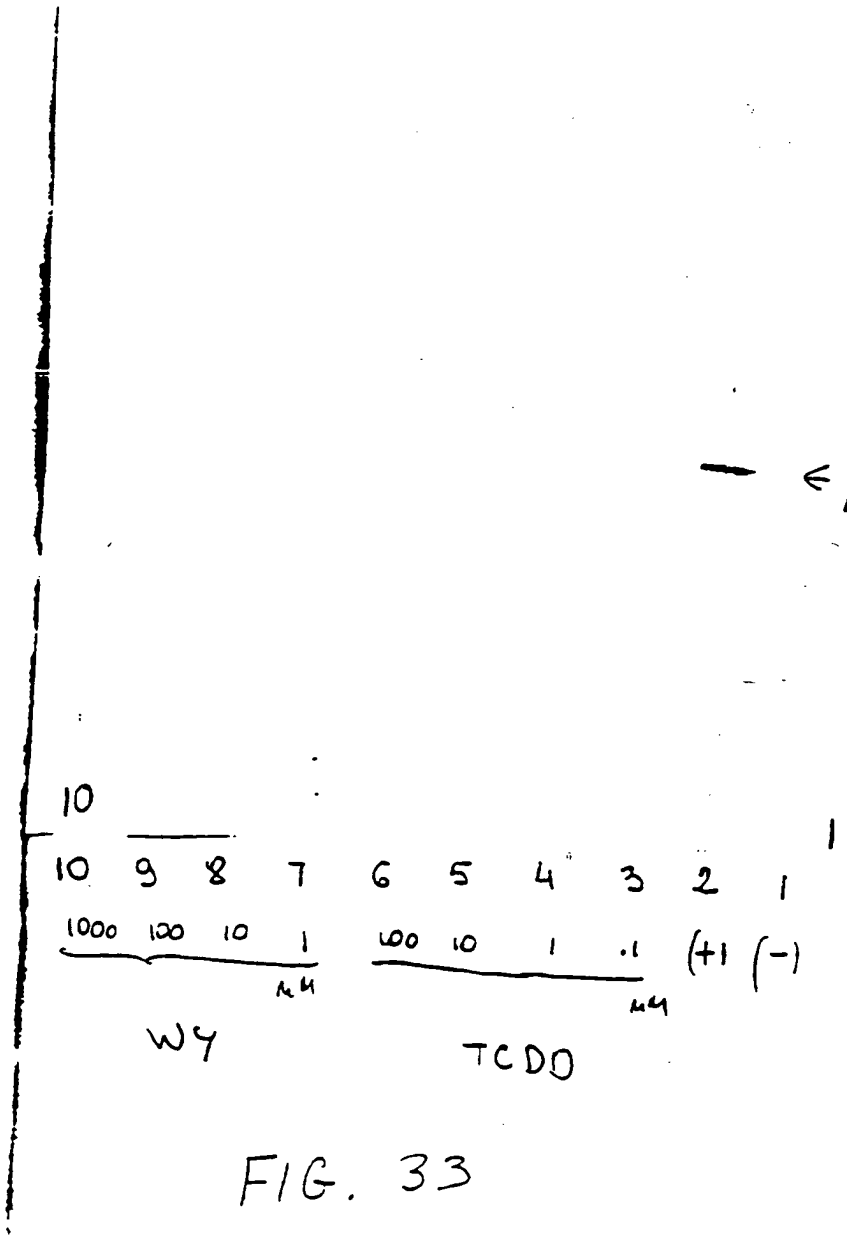
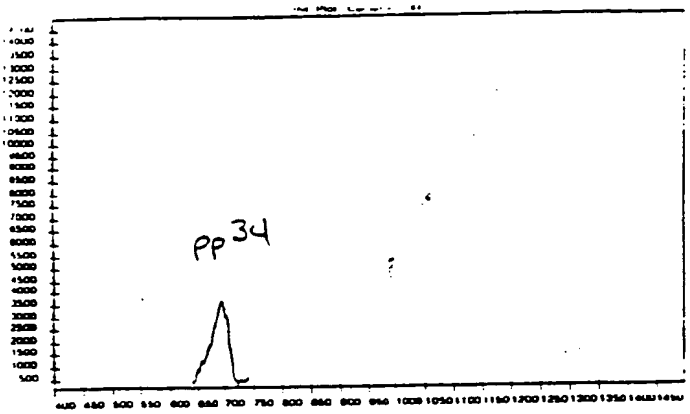
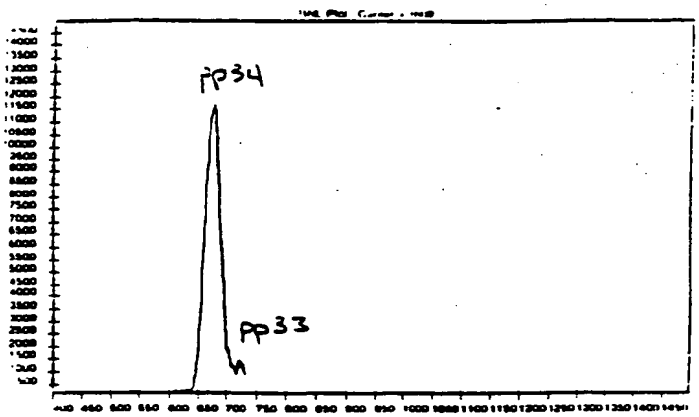
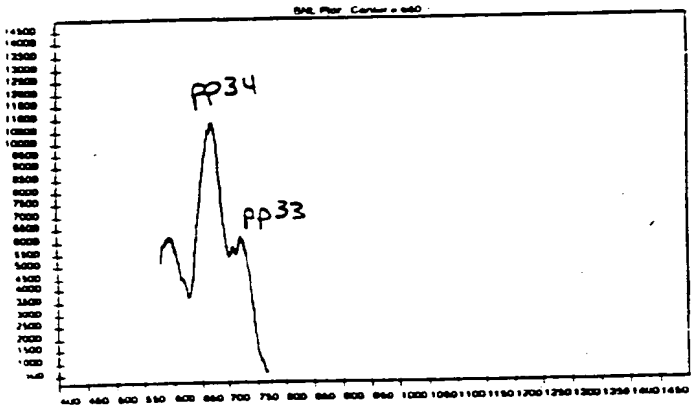
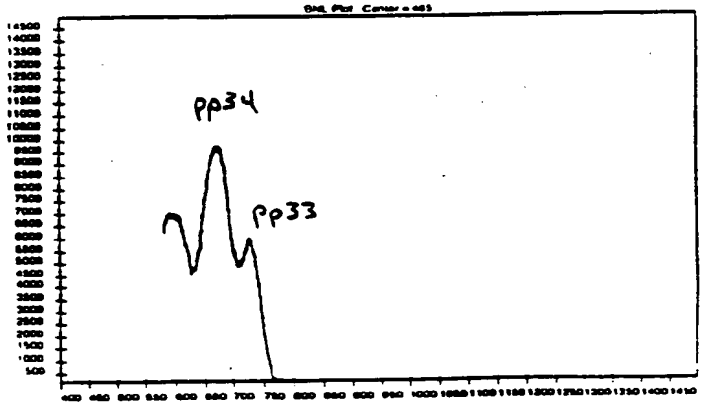


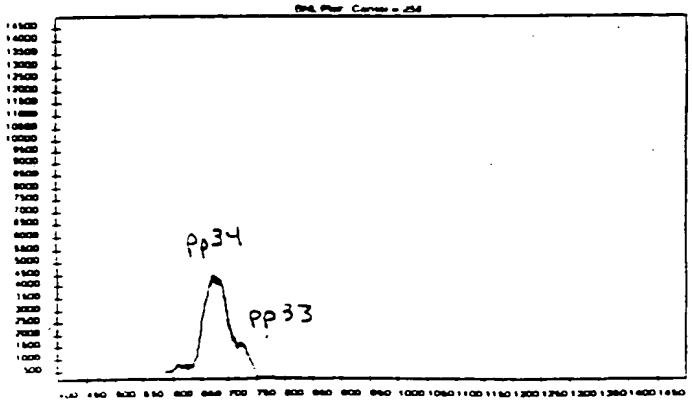
FIG. 33

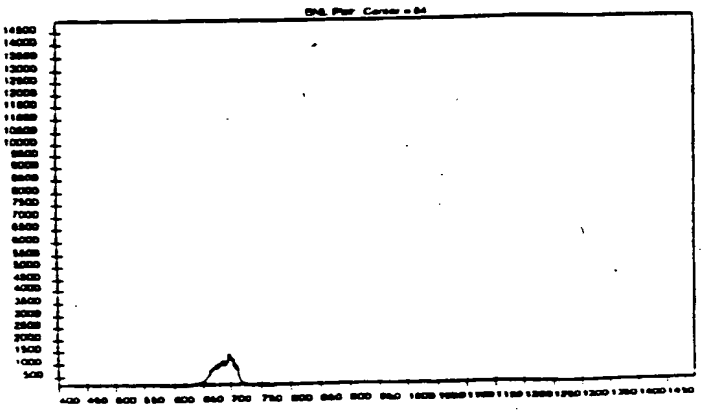

pp34


pp34
pp33


pp34
pp33


pp34
pp33


pp34
pp33



35/40

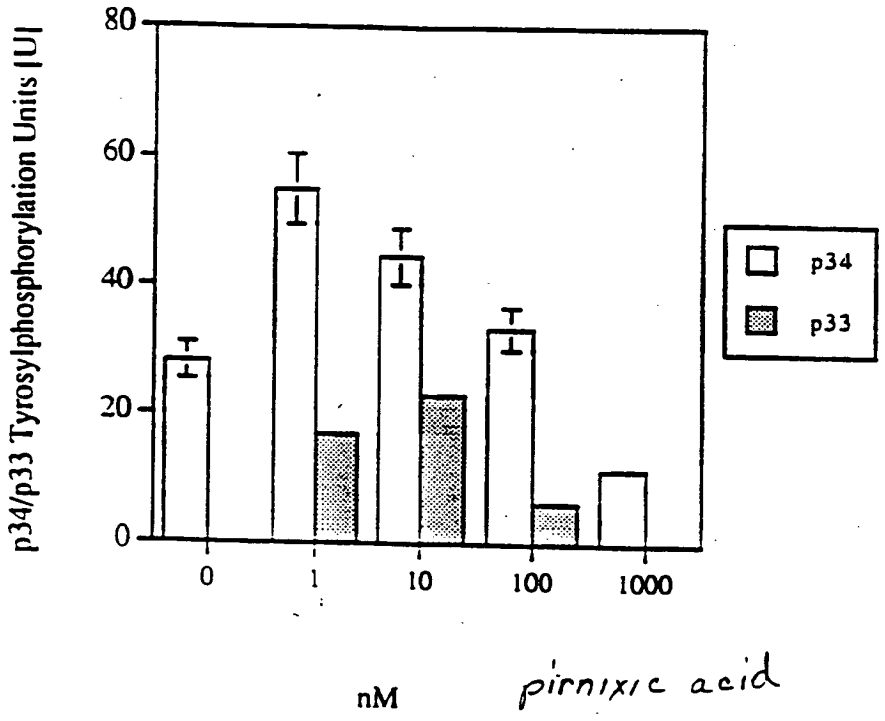
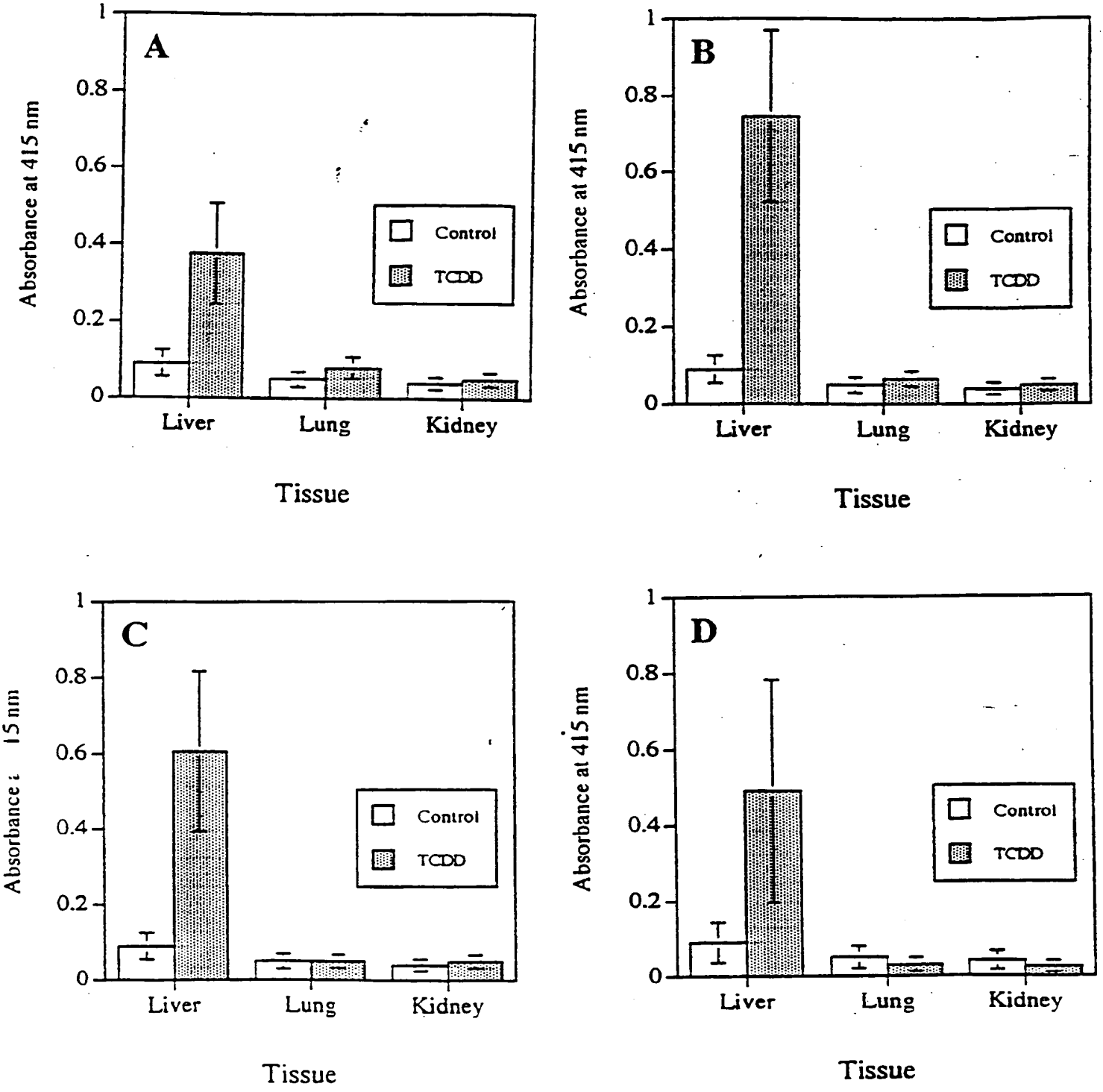

p34/p33 Tyrosylphosphorylation Units [U]
80
60
40
20
0
0
1
10
100
1000
nM
p34
p33
pirnixic acid

FIG 35


A
B
C
D
Absorbance at 415 nm
1
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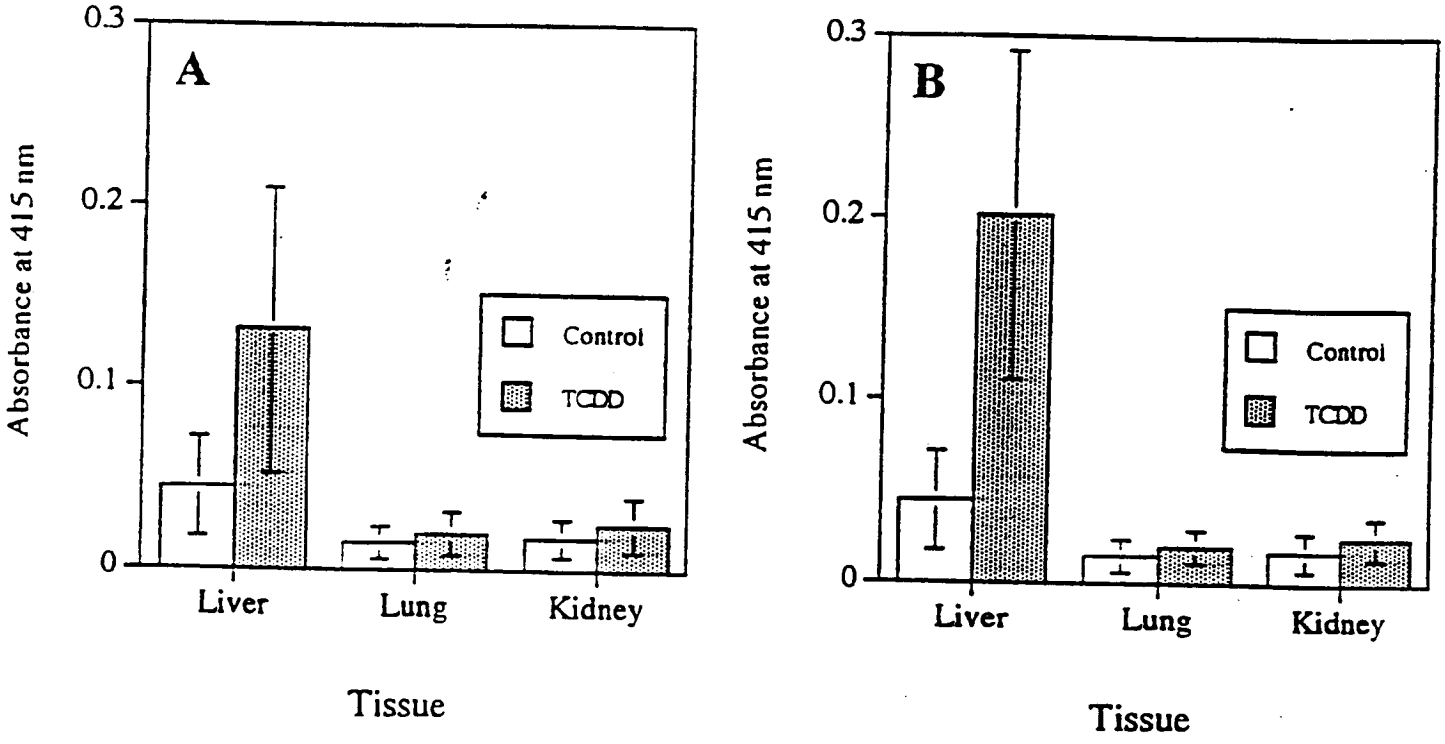
0.6
0.4
0.2
0
Control
TCDD
Liver
Lung
Kidney
Tissue

FIG. 36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B
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Absorbance at 415 nm
Control
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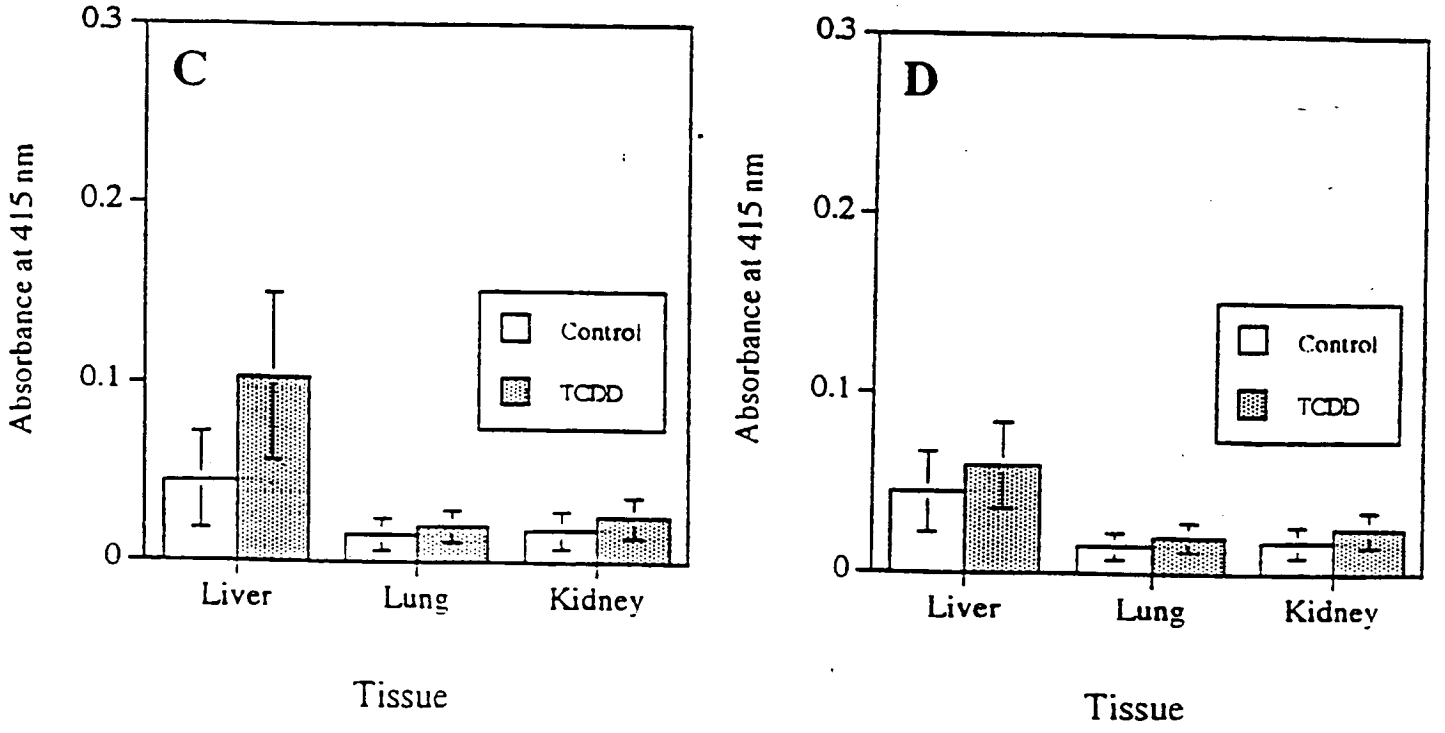
Liver
Lung
Kidney
Tissue


C
D
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0.1
0
Absorbance at 415 nm
Control
TCDD
Liver
Lung
Kidney
Tissue

FIG. 37

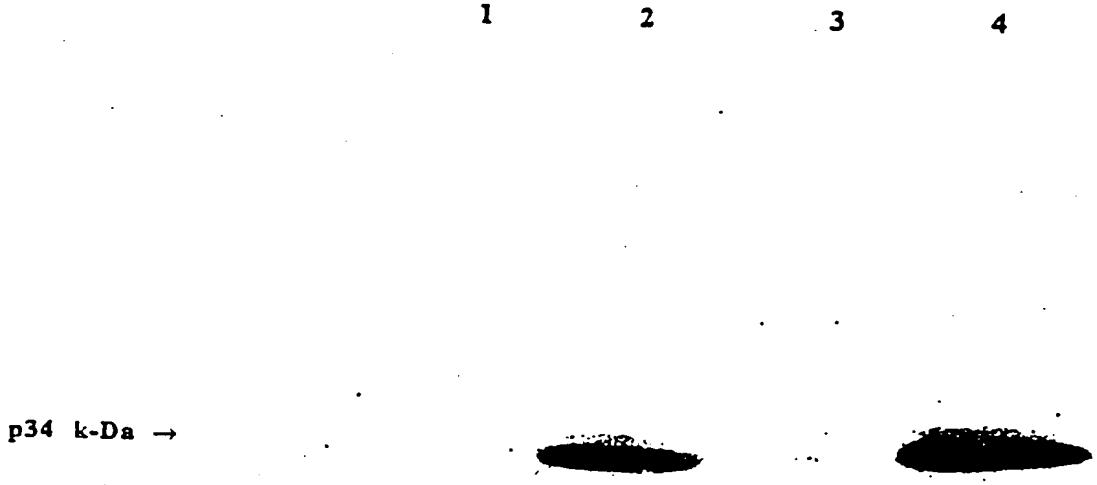


Fig 38

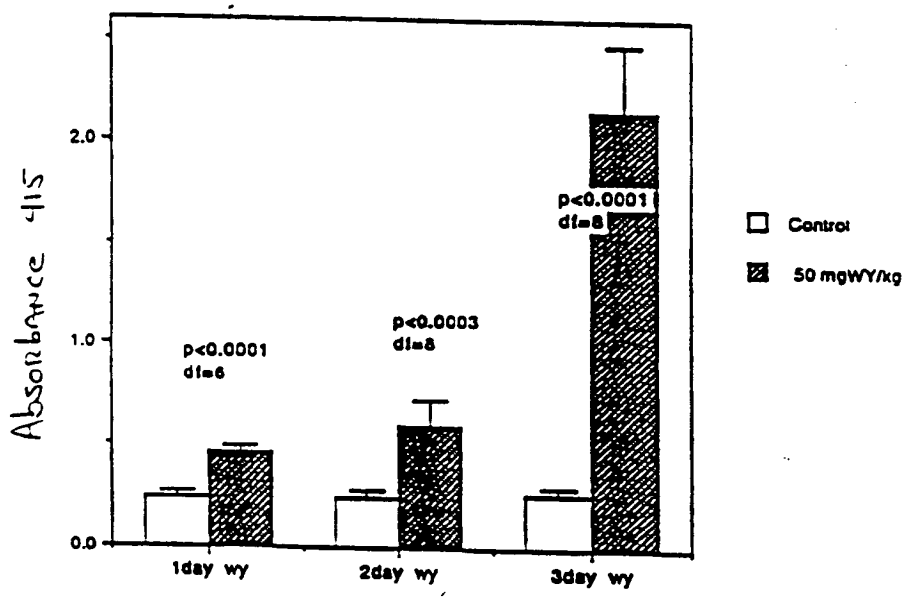
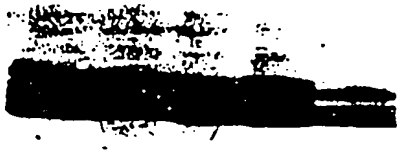

Absorbance 415
2.0
1.0
0.0
p<0.0001
df=6
p<0.0003
df=8
p<0.0001
df=8
1day wy
2day wy
3day wy
Control
50 mgWY/kg

Fig 39

40/40



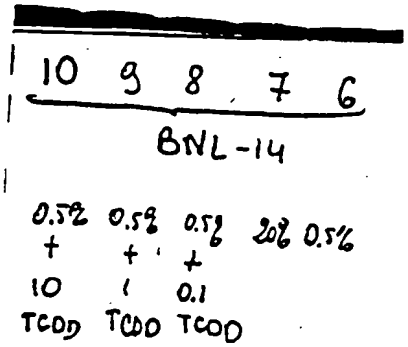


FIG 40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00961

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/574, 33/573, 33/53; C12Q 1/50
US CL :435/7.23, 7.4, 7.9, 17; 436/64, 813
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.23, 7.4, 7.9, 17; 436/64, 813

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG: Medline, Biosis, Embase, Cancerlit, Derwent; APS
search terms: cyclin, kinase, cancer, carcinoma, malig?, neoplas?, transform?, p34cdc2, dependent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	Cancer Research, Volume 53, No. 22, issued 15 November 1993, Z.A. Khatib et al, "Coamplification of the *CDK4* Gene with *MDM2* and *GLI* in Human Sarcomas", p. 5535-5541. See the abstract, the right-hand column of page 5535, the right hand column of page 5539, and the left-hand column of page 5540.	1-45
Y, P	Cancer Research, Volume 53, No. 3, issued 01 February 1993, S. Mishra et al, "*O*-Phospho-L-tyrosine Inhibits Cellular Growth by Activating Protein Tyrosine Phosphatases", pages 557-563. See the Abstract.	1-45

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00961

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Annu. Meet. Am. Asso. Cancer Res., Volume 33, issued 1992, M.J. Marcote et al, "Mechanisms of Activation of Cyclin-Dependent Kinases", Abstract No. A575, see the entire abstract.	1-45
Y, P	Proc. Annu. Meet. Am. Assoc. Cancer Res., Volume 34, issued 1993, M.R. Hellmich et al, "The Kinase CDK5 is Expressed in Breast, Lung and Prostate Tumor Cells in Contrast to Its Selective Expression in Postmitotic Neurons", Abstract No. A244, see the entire abstract.	1-45

CORRECTED VERSION*

PCT

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Filed on 11 June 1993 (11.06.93)

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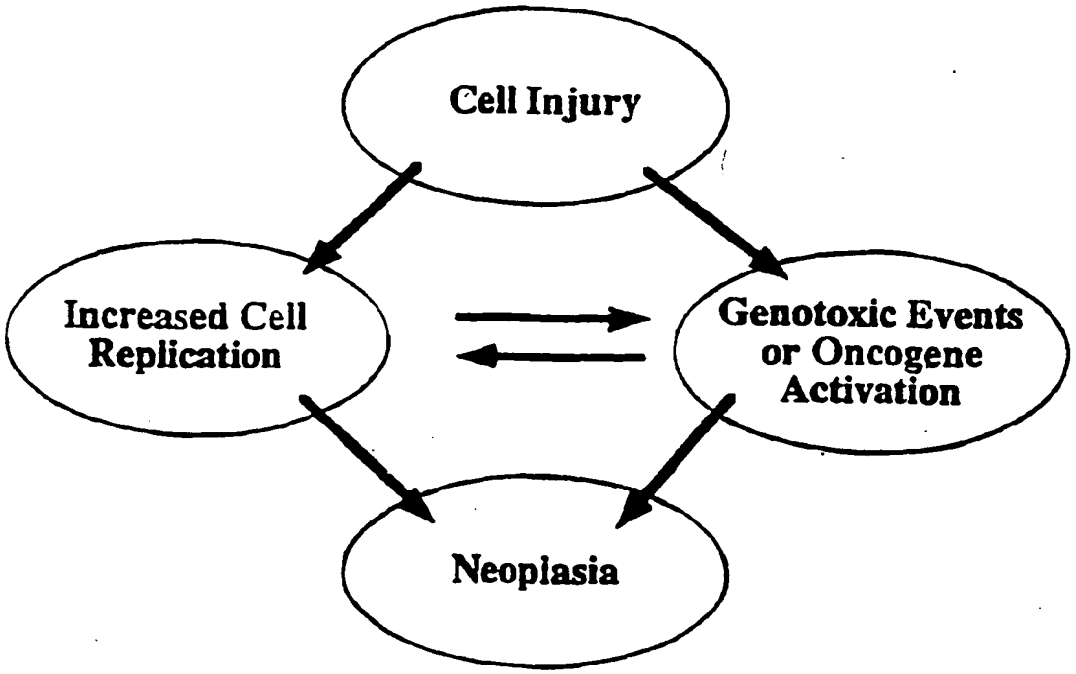
(54) Title: PRODUCTS FOR MEASURING CELL GROWTH PROPENSITY AND METHODS FOR THEIR USE



(57) Abstract

In vivo and (in vitro) assays are disclosed for measuring cyclin dependent kinase concentrations in cells or tissues, methods are provided for their use, including the use of such assays to evaluate carcinogenicity of a test compound, potential antineoplastic agents, and effectiveness of regions for increasing cell growth.

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PRODUCTS FOR MEASURING CELL GROWTH PROPENSITY AND METHODS FOR THEIR USE

This is a continuation-in-part application of U.S. Patent Application Serial No. 08/075,744, filed June 11, 1993, which in turn is a continuation-in-part application of U.S. Patent Application Serial No. 08/007,636, filed January 21, 1993.

FIELD OF THE INVENTION

This invention relates to *in vivo* and *in vitro* assays for measuring cell growth propensity and numerous applications therefor, including but not limited to the detection and quantification of substances, which are carcinogenic, even if the substances exhibit negative results in genotoxicity or mutagenicity tests.

BACKGROUND OF THE INVENTION

Introduction

Cell proliferation is the most fundamental phenotypic property of cancer. The stimulus for cellular proliferation is central not only at the late steps in carcinogenesis, the cancer, but also at the earliest known step, initiation (1,2) and Figure 1. In fact, cell proliferation exerts an influence in the initiation of carcinogenesis in that cells in the S phase are more sensitive toward many initiators than at other times in the cell cycle (3). A myriad of short-term tests exist for the assessment of the carcinogenic potential of

chemicals. These tests detect only carcinogens that interact with nucleic acids, or induce DNA repair synthesis or mutations in bacterial or mammalian cells (4-8).

As testing of the genotoxicity and carcinogenicity of chemicals has become routine, a growing number of compounds have been found to induce tumors in chronic bioassays while exhibiting negative results in genotoxicity tests (9). Significant examples of these classes of compounds include the dioxins, chlorinated biphenyls and peroxisome proliferators. These chemicals are often active as tumor promoters in two-stage experiments and exhibit biological activities as hormones (ethinylestradiol), peroxisome proliferators (pirnixic acid) or enzyme inducers (phenobarbital) (10).

At the present time only the initiation-promotion assay is employed routinely. In this assay the test compounds are examined for their ability to promote hepatic tumors or foci formation after initiation with a known genotoxic agent (11,12). As currently formatted, this assay utilizes animals, requires several months to perform, and produces histological endpoints that are difficult to quantify and do not lend to rigorous dose-response calculations for the purposes of risk assessment (13).

Stimulation of DNA synthesis has been proposed as an assay for short-term assessment of nongenotoxic carcinogens and tumor promoters *in vivo* (14,15). This methodology has potential for application to routine testing. So far, only one result has been detected that is inconsistent with carcinogenicity bioassay data. The different carcinogenicity of di(2-ethylhexyl)adipate (negative in rats) and di(2-ethylhexyl)phthalate

(positive) was not detectable by DNA stimulation index using ^{3}H-thymidine. Both plasticizers were positive in this short-term system with doubling doses of 0.7 mmol/kg for di(2-ethylhexyl)adipate and 0.5 mmol/kg for di(2-ethylhexyl)phthalate. Other disadvantages of this system include the use of radioactivity and the high coefficient of variation in the endpoint.

Several *in vitro* models have been utilized for the assessment of nongenotoxic carcinogens. Chida et al. (16) modeled the activation of protein kinase C and specific phosphorylation of a 90,000 kDa membrane protein of promotable BALB/3T3 and C3H/10T1/2 cells by tumor promoters. Smith and Colburn also utilized protein kinase C and its substrates in tumor promoter-sensitive and tumor-resistant cells as a biochemical marker for the response of cells to tumor promoters (17). However, these systems were flawed by both false positive and false negative values. The false positive values may be due to the fact that the activation of protein kinases C represents a biochemical signal far upstream from the final proliferative signal. While the false negatives may result from the fact that protein kinase C represents only a single receptor-mediated response. At least four other receptor responses, which are independent of protein kinase C, are known for tumor promotion and activity of nongenotoxic carcinogens (e.g. dioxin receptor, peroxisome proliferator receptor, phenobarbital receptor and estrogen receptor) (14,18).

Protein tyrosine phosphorylation

Protein-tyrosine kinases (PTK) constitute a class of enzymes that catalyze the transfer of the μ-

of proto-oncogenes (25) and their aberrant expression has been associated with a variety of human cancers (26).

The cascade of protein tyrosine phosphorylation following the activation of protein tyrosine kinases appears to regulate the proliferative response (27,28). Specific, protein tyrosylphosphorylations are common to a wide variety of nongenotoxic carcinogens independent of associated receptors or known mechanism of action. The present invention demonstrates the xenobiotic alterations in protein tyrosine phosphorylation at a fundamental point in the control of cellular proliferation and on an assay protocol that characterizes the ability of a xenobiotic test chemical to initiate cellular proliferation.

Cyclin-dependent Kinases (CDK)

Recent experimental evidence suggests that the cell cycle of all eukaryotic cells is controlled at several checkpoints by different members of a novel class of protein kinase, the cyclin-dependent kinases (29, 31, 36, 46). The most well known of these kinases is the 34 kD product of the cdc2 gene in the fission yeast $p34^{cdc2}$; however, several putative cyclin-dependent kinases (CDK) have now been cloned or identified. Some of these clones resemble $p34^{cdc2}$.

At least nine CDKs have been described in the literature; these all have a common PSTAIR epitope. Therefore anti-PSTAIR would be expected to cross react with the entire complement of CDKs showing up in the 32 to 34 kD region. (Apparently some cyclins also cross react with the anti-PSTAIR antibody and this explains the banding at approximately 60 kD observed in some of the immunoblots with anti-PSTAIR.)

The antibody to the C-terminus region is more specific for $p34^{cdc2}$ kinase, since the C-terminus region is more variable than the highly conserved PSTAIR region. However, it is obviously not species-specific since it was generated against human cdc2 and it cross reacts with mouse, rat and dog $p34^{cdc2}$ kinase.

SUMMARY OF THE INVENTION

In one embodiment, the invention provides a method for determining growth propensity for a tissue sample or cell line, said method comprising measuring a parameter that is indicative of concentration, in said sample or cell line, of at least one cyclin dependent kinase, and correlating said growth propensity to said measurement. Kits for carrying out this method are also provided.

Another embodiment of the invention provides a diagnostic method for determining whether a tissue or cell sample has undergone transformation to a cancerous phenotype, said method comprising measuring a parameter indicative of concentration, in said tissue or cell sample, of at least one cyclin dependent kinase, and correlating said measurement to likelihood of transformation

Another embodiment of the invention provides a diagnostic method for determining a likelihood that a tissue or cell sample will undergo transformation to a cancerous phenotype, said method comprising measuring a parameter that is indicative of concentration, in said tissue or cell sample, of at least one cyclin dependent kinase, and correlating said likelihood to said measurement.

Another embodiment of the invention provides a method of measuring carcinogenicity of a test substance comprising contacting said test substance with cells or tissue capable of expressing cyclin dependent kinase and thereafter measuring a parameter indicative of concentration, in said cells or tissue, of at least one cyclin dependent kinase, and correlating said carcinogenicity with said measurement.

Another embodiment of the invention provides a method of measuring effectiveness of a putative antineoplastic agent comprising the steps of:

(A) providing a sample of transformed cells;

(B) contacting said transformed cells with said putative antineoplastic agent;

(C) measuring a parameter indicative of concentration, in said cells, of at least one cyclin dependent kinase; and

(D) determining whether, or to what extent, said measurement indicates a decrease in cyclin dependent kinase following step (B).

Another embodiment of the invention provides a kit for measuring cyclin dependent kinase concentration in human or animal cell lysates, said kit including antibodies to an antigen whose concentration is

indicative of cyclin dependent kinase concentration in said lysates.

Another embodiment of the invention provides an immunohistochemistry kit for determining whether cells or tissues have undergone transformation to a cancerous phenotype or are likely to undergo such transformation, said kit comprising a slide for receiving a thin tissue slice containing said cells and further comprising an antibody to an antigen whose concentration is indicative of concentration of at least one cyclin dependent kinase in said cell or tissue sample.

Another embodiment of the invention provides a method for determining efficacy of a regimen for reducing or enhancing cell growth, said method comprising the steps of measuring a parameter indicative of concentration levels of at least one cyclin dependent kinase following treatment of those cells with said regimen and correlating cyclin dependent kinase concentration with said efficacy.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic of the multistage nature of carcinogenesis, from carcinogen exposure through initiation, promotion, conversion and progression. Nongenotoxic carcinogens and tumor promoters affect, respectively, defects in terminal differentiation and selective clonal expansion of initiated cells. Initiation can be caused, inter alia, by exposure to radiation or chemical or viral carcinogens. Genetic change can be accompanied by activation of proto-oncogenes and/or inactivation of tumor suppressor genes. Genetic changes can be accompanied by defects in

terminal differentiation, defects in growth control, and/or resistance to cytotoxicity.

Figure 2. A representation of the relationship between cell injury and neoplasia. The role of cell proliferation is characterized by the increased cell replication step.

Figure 3. The cell cycle. A cell can either be quiescent or continue to grow. The decision point is early in the G1 phase when a cell either passes START -

and then is committed to growing, finishing the rest of the cycle and dividing (G1, S, G2 and M) - or the cell enter the G_0 state in which it continues to metabolize but does not grow.

Figure 4. Immunoblot using anti-PSTAIR antibody. An anti-phosphotyrosine immunoprecipitate of the murine hepatic S-9 protein is separated using an 11% SDS-PAGE gel. The separated proteins are transferred to a blotting membrane and probed with the anti-PSTAIR antibody.

Figure 5. Scanning densitometry of anti-PSTAIR immunoblots for hepatic S-9 fraction of 2,3,7,8-tetrachlorodibenzo-p-dioxin treated female, C57BL/6J mice.

Figure 6. Bar graph depicting the quantification of the results of the scanning densitometry. The cyclin dependent kinase (CDK) quantified from the anti-PSTAIR immunoblot was at 32 kDa. The administration of a single dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin results in enhanced tyrosylphosphorylation of the CDK compared to control animals, which exhibit no tyrosylphosphorylation of CDK. Each group on the graph represents the single result of scanning an anti-PSTAIR immunoblot produced from the pooled hepatic S-9 of three animals. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 7. A typical BIAcore® sensorgram produced on immobilization of anti-cdc2 kinase C-terminus.

Figure 8. Anti-phosphotyrosine immunoblots of rat hepatic S-9 protein separated using 11% SDS-PAGE gels

for pirnixic acid-treated (lanes 1,2) and control (3,4) rats. Each lane represents a single rat.

Figure 9. Scanning densitometry of anti-phosphotyrosine immunoblots for pirnixic acid-treated rats [A and B] and paired vehicle controls [C and D, respectively]. Bolding of peaks indicates difference of greater than 40 percent between treatment and control.

Figure 10. Bar graph depicting the quantification of the results of the scanning densitometry. The phosphotyrosyl protein quantified from the anti-phosphotyrosine immunoblot was at 33 kDa. Results indicate that the administration of five, twice-daily doses of pirnixic acid (50 mg/kg each dose) produces enhanced tyrosylphosphorylation of p33 compared to control animals, which exhibit no tyrosylphosphorylation at 33 kDa. Each group on the graph represents the average of two rats. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 11. BIAcore® sensorgram displaying binding of pirnixic acid-treated S-9 protein and control S-9 protein over immobilized anti-cdc2 PSTAIR monoclonal antibody.

Figure 12. Summary bar graph depicting BIAcore® quantification of the interaction of tyrosylphosphorylated cyclin dependent kinases (CDK) with anti-CDK monoclonal antibodies (PSTAIR and C-terminus) from control and pirnixic acid-treated rats. Error bars represent standard deviations of n = 6 (anti-PSTAIR) and n = 8 (anti-C Terminus) control rats. RU (response units) value for pirnixic acid-treated rats represents the mean of 2 animals. The treatment of rats with 50 mg pirnixic acid/kg twice a day for 5 days results in

enhanced tyrosylphosphorylation of CDK ($p34^{cdc2}$ kinase) compared to control rats.

Figure 13. Anti-phosphotyrosine immunoblots of rat hepatic S-9 protein separated using 11% SDS-PAGE gels for diethylhexylphthalate-treated (lanes 1,2) and control (3,4) rats. Each lane represents a single rat.

Figure 14. Scanning densitometry of anti-phosphotyrosine immunoblots for diethylhexylphthalate-treated rats [A and B] and paired vehicle controls [C and D, respectively]. Bolding of peaks indicates difference of greater than 40 percent between treatment and control.

Figure 15. Bar graph depicting the quantification of the results of the scanning densitometry. The phosphotyrosyl protein quantified from the anti-phosphotyrosine immunoblot was at 34 kDa. Results indicate that the administration of five, twice-daily doses of diethylhexylphthalate (500 mg/kg each dose) produces enhanced tyrosylphosphorylation of the p34 compared to control animals, which exhibit no tyrosylphosphorylation at 34 kDa. Each group on the graph represents the average of two rats. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 16. Summary bar graph depicting BIAcore® quantification of the interaction of tyrosylphosphorylated cyclin dependent kinases (CDK) with anti-CDK monoclonal antibodies (PSTAIR and C-terminus) from control and diethylhexylphthalate-treated rats. Error bars represent standard deviations of n = 6 (anti-PSTAIR) and n = 8 (anti-C Terminus) control rats. RU value for diethylhexylphthalate-treated rats represents the mean of 2 animals. The treatment of rats with 500 mg diethylhexylphthalate/kg twice a day for 5 days produces

enhanced tyrosylphosphorylation of CDK ($p34^{cdc2}$ kinase) compared to control rats.

Figure 17. Anti-phosphotyrosine immunoblots of rat hepatic S-9 protein separated using 11% SDS-PAGE gels for diethylnitrosamine-treated (lanes 3,4) and control (lanes 1,2) rats.

Figure 18. Scanning densitometry of anti-phosphotyrosine immunoblots for diethylnitrosamine-treated rats [A and B] and paired vehicle controls [C and D, respectively]. Bolding of peaks indicates difference of greater than 40 percent between treatment and control.

Figure 19. Bar graph depicting the quantification of the results of the scanning densitometry. The phosphotyrosyl protein quantified from the anti-phosphotyrosine immunoblot was at 34 kDa. Results indicate that the administration of five, twice-daily doses of diethylnitrosamine (500 mg/kg each dose) produces no enhanced tyrosylphosphorylation of p34 compared to control animals. Each group on the graph represents the average of two rats. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 20. Summary bar graph depicting BIAcore® quantification of the interaction of tyrosylphosphorylated cyclin dependent kinases (CDK) with anti-CDK polyclonal antibodies (PSTAIR and C-terminus) from control and diethylnitrosamine-treated rats. Error bars represent standard deviations of n = 6 (anti-PSTAIR) and n = 8 (anti-C-terminus) control rats. RU value for diethylnitrosamine-treated rats represents the mean of 2 animals. Results indicate that the treatment of rats with 500 mg diethylnitrosamine/kg twice a day for 5 days

produces no enhanced tyrosylphosphorylation of CDK ($p34^{cdc2}$ kinase) compared to control rats.

Figure 21. Anti-phosphotyrosine immunoblot of dog hepatic S-9 protein separated using 11% SDS-PAGE gels for Aroclor®-treated dogs. Lanes 1, 2, 3, 4 and 5 are control, 0.6, 0.8, 4 - 8, and 5 - 10 mg Aroclor®/kg-day, respectively.

Figure 22. Scanning densitometry of anti-phosphotyrosine immunoblots at 34 kDa for Aroclor®-treated dogs. Figures 22(a)-22(e) represent 0.6, 0.8, 4 - 8, and 5 - 10 mg Aroclor®/kg-day, respectively.

Figure 23. Bar graph depicting the quantification of the scanning densitometry of the putative cyclin dependent kinase (p34) from the anti-phosphotyrosine immunoblot. The daily administration of Aroclor® for a period of 11.5 weeks results in enhanced tyrosylphosphorylation of the p34 at all doses compared to the control dog. Each bar on the graph represents the result of scanning an immunoblot produced from the hepatic S-9 of a single dog. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 24. Anti-phosphotyrosine immunoblots of 3T3 cell lysate protein separated using 11% SDS-PAGE gels for 3T3 cells exposed to 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (lane 3B) or DMSO vehicle (lane 1B) for 24 h in 0.5% serum supplemented media.

Figure 25. Scanning densitometry of anti-phosphotyrosine immunoblots for 3T3 cells treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or DMSO vehicle (Control) for 24 h in 0.5% serum media. Bolded peaks indicate p34 and p33 tyrosylphosphoproteins.

Figure 26. Bar graph depicting the quantification of the scanning densitometry of the putative cyclin dependent kinases (p34/p33) from the anti-phosphotyrosine immunoblot. Exposure of 3T3 cells to 10 nM 2,3,7,8-tetracholordibenzo-p-dioxin for 24 h results in an increase in tyrosylphosphorylation of p34 and p33 of 67 and 32%, respectively, compared to the vehicle control. Each bar on the graph represents the result of scanning an immunoblot produced from the pooled whole cell lysates of four plates per treatment. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 27. Anti-phosphotyrosine immunoblots of 3T3 cell lysate protein separated using 11% SDS-PAGE gels for 3T3 cells exposed to 160 nM 12-O-tetra-decanoylphorbol-13-acetate (TPA; lane 4B) or DMSO vehicle (Control; lane 1B) for 24 h in 0.5% serum supplemented media.

Figure 28. Scanning densitometry of anti-phosphotyrosine immunoblots for 3T3 cells treated with 160 nM 12-O-tetra-decanoylphorbol-13-acetate (TPA) or DMSO vehicle for 24 h in 0.5% serum media. Bolded peaks indicate p34 and p33 tyrosylphosphoproteins.

Figure 29. Bar graph depicting the quantification of the scanning densitometry of the putative cyclin dependent kinases (p34/p33) from the anti-phosphotyrosine immunoblot. Exposure of 3T3 cells to 160 nM 12-O-tetra-decanoylphorbol-13-acetate (TPA) for 24 h results in an increase in tyrosylphosphorylation of p34 and p33 of 54 and 95%, respectively, compared to the vehicle control. Each bar on the graph represents the result of scanning an immunoblot produced from the pooled whole cell lysates of four plates per treatment. Error

bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 30. Anti-phosphotyrosine immunoblots of BNL CL.2 cell lysate protein separated using 11% SDS-PAGE gels for BNL CL.2 cells exposed to 0.1, 1, 10, or 100 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; lanes 3,4,5 and 6, respectively) or DMSO vehicle (lane 1) for 24 h in 0.5% serum supplemented media. Lane 2 is the 20% serum-supplemented control.

Figure 31. Scanning densitometry of anti-phosphotyrosine immunoblots in the 35 to 30 kDa molecular weight range for BNL CL.2 cells treated with 0.1, 1, 10 or 100 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or DMSO vehicle (Control) for 24 h in 0.5% serum supplemented media. p34 and p33 tyrosylphosphoproteins are indicated for the respective treatments. Figures 31(a) and 31(b) are respectively 0.5 % and 20% serum supplementation; Figures 31(c) and 31(d) are respectively 0.1 and 1 nM TCDD; Figures 31(e) and 31(f) are respectively 10 and 100 nM TCDD.

Figure 32. Bar graphs depicting the quantification of the scanning densitometry of the putative cyclin dependent kinases (p34-top/p33-bottom) from the anti-phosphotyrosine immunoblot. Exposure of BNL CL2 cells to 0.1, 1, 10 or 100 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for 24 h results in a similar increase in tyrosylphosphorylation of p34, averaging 180% of the vehicle control over all concentrations of TCDD. Twenty percent serum supplementation results in an increase of tyrosylphosphorylation of p34 of 229% of the vehicle control. Vehicle controls at 0.5% serum supplementation exhibit no tyrosylphosphorylation at p33, while TCDD exposure at the four concentrations enhances

tyrosylphosphorylation of this putative CDK to 0.9, 2.0, 2,0 and 1.9 density units, respectively. The increases in tyrosylphosphorylation of p33 by TCDD are 3.4 times the p33 tyrosine phosphorylation produced by 20% serum supplementation. Each bar on the graph represents the result of scanning an immunoblot produced from the pooled whole cell lysates of four plates per treatment. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 33. Anti-phosphotyrosine immunoblots of BNL CL.2 cell lysate protein separated using 11% SDS-PAGE gels for BNL CL.2 cells exposed to 1, 10, 100, or 1000 nM pirnixic acid (lanes 7,8,9 and 10, respectively) or DMSO vehicle (lane 1) for 24 h in 0.5% serum supplemented media. Lane 2 is the 20% serum-supplemented control.

Figure 34. Scanning densitometry of anti-phosphotyrosine immunoblots in the 35 to 30 kDa molecular weight range for BNL CL.2 cells treated with 1, 10, 100, or 1000 nM pirnixic acid or DMSO vehicle (Control) for 24 h in 0.5% serum media. p34 and p33 tyrosylphosphoproteins are indicated for the respective treatments. Figures 34(a) and 34(b) are respectively 0.5 % serum and 20% serum; Figures 34(c) and 34(d) are respectively 1 and 10 nM pirnixic acid; Figures 34(e) and 34(f) are respectively 100 and 1000 nM pirnixic acid.

Figure 35. Bar graphs depicting the quantification of the scanning densitometry of the putative cyclin dependent kinases (p34-top/p33-bottom) from the anti-phosphotyrosine immunoblot. Exposure of BNL CL2 cells to pirnixic acid for 24 h results in

increases in tyrosylphosphorylation of p34 relative to the vehicle control for the 1 and 10 nM concentrations, 96 and 58% increases, respectively. At 100 nM pirnixic acid, the tyrosylphosphorylation of p34 is similar to the

vehicle control, while at 1000 nM tyrosine phosphorylation of p34 is depressed 60% from the vehicle control. Twenty percent serum supplementation results in an increase of tyrosylphosphorylation of p34 of 229%, relative to the vehicle control. The 0.5% serum supplementation control exhibits no tyrosylphosphorylation at p33, while pirnixic acid exposure enhances tyrosylphosphorylation of this putative CDK to 2.0, 2.5 and 0.5 density units, respectively, at the 1, 10, and 100 nM concentrations. The increases in tyrosylphosphorylation of p33 by pirnixic acid at 1 and 10 nM are roughly 4 times the p33 tyrosine phosphorylation produced by 20% serum supplementation. Each bar on the graph represents the result of scanning an immunoblot produced from the pooled whole cell lysates of four plates per treatment. Error bars represent the 10 percent coefficient of variation in the quantification of density.

Figure 36. Bar graph depicting the microtiter methodology for quantification of tyrosylphosphorylation of tissue CDK. The capture antibody was anti-PSTAIR and the secondary antibody was anti-phosphotyrosine. Dosing of C57BL/6J female mice daily with 0, 0.25, 0.5, 1 or 2 ng TCDD/kg-day (A, B, C and D, respectively) results in enhanced tyrosylphosphorylation of hepatic CDK but not pulmonary or renal CDK. This identifies the target tissue for the cellular proliferative effects of TCDD as the liver. Maximal increase in tyrosylphosphorylation of hepatic CDK is observed at the 0.5 ng TCDD/kg-day dose regimen. The error bars represent the 95 percent confidence interval of the mean absorbance determined at 415 nm for each of the treatments (n=10 mice per treatment).

Figure 37. Bar graph depicting the microtiter methodology for quantification of tyrosylphosphorylation of tissue $p34^{cdc2}$ kinase. The capture antibody was anti-C-terminus and the secondary antibody was anti-phosphotyrosine. Dosing of C57BL/6J female mice daily with 0, 0.25, 0.5, 1 or 2 ng TCDD/kg-day (A, B, C and D, respectively) results in enhanced tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase but not pulmonary or renal $p34^{cdc3}$ kinase. This identifies the target tissue for the cellular proliverative effects of TCDD as the liver. Maximal increase in tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase is observed at the 0.5 ng TCDD/kg-day dose regimen. The error bars represent the 95 percent confidence interval of the mean absorbance determined at 415 nm for each of the treatments (n=10 mice per treatment).

Figure 38. The anti-cdc2 C-terminus immunoblot of rat hepatic S9 proteins separated using 10 to 11% SDS-PAGE gels for control (lanes 1 and 3) and WY 14,643-treated rats (lanes 2 and 4). A single intensely-stained band was visible in the CDK region (32 to 35 kDa) in hepatic S9 samples obtained from rats three days after receiving a single does of 50 mg WY14,643/kg. This band is barely visible in hepatic S9 from control rats.

Figure 39. Bar graph depicting the microtiter methodology for quantification of CDK expression in rat liver S9. The treated rats receive a single does of 50 mg pirnixic acid/kg and are killed 1, 2 or 3 days later; control rats are dosed with the vehicle alone. The mean absorbance developed at 415 nm over 10 min is presented on the y-axis. Error bars represent standard deviations of n = 4 (1 day) and n = 5 (2 and 3 day) rats per treatment. The extent of CDK expression the livers

of young, male rats receiving a single does of 50 mg/kg of WY 14,643 increases steadily during the 3-day postdosing observation period. CDK expression in control animals remains constant over the same 3-day period.

Figure 40. Anti-cdc2 C-terminus immunoblot of BNL CL.2 cell lysate protein separated using 10 to 11% SDS-Page gels for BNL CL.2 cells exposed to 0.1, 1, or 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioin (TCDD; lanes 8, 9, and 10, respectively) or DMSO vehicle (lane 6) for 48 h in 0.5% serum supplemented media. Lane 7 is the 20% serum-supplemented control. TCDD exposure results in increased expression of CDK relative to the DMSO control.

DETAILED DESCRIPTION OF CERTAIN PREFERRED EMBODIMENTS

In accordance with the present invention, there is provided novel methods, and kits for performing the methods, for measuring parameters indicative of the concentration of at least one cyclin dependent kinase in human or animal tissues, cell lines, cell lysates, tissue homogenates and the like. Applicants have discovered a relationship between cyclin dependent kinase concentration and cell growth (or propensity therefor). Cell proliferation being the most fundamental phenotypic property of cancer, the present invention has broad application to, *inter alia*, determining whether cells or tissue have transformed to a cancerous phenotype, determining the likelihood of such transformation later occurring, detecting and quantifying carcinogenicity of test substances (even substances which are nongenotoxic and/or nonmutagenic), testing putative antineoplastic agents, etc.

The invention also has broader applications in determining cell growth in general, and in evaluating the

effectiveness of regimens designed to increase or decrease cell growth. Without intending to be bound by theory, it is believed that the concentration of cyclin dependent kinase is indicative of the proportion of cells which are out of the G. phase of their cell cycle. Thus, measuring cyclin dependent kinase concentration (or a related parameter) provides a very early indication of increased cell growth (or a propensity therefor) significantly sooner than cell growth or cell transformation can be observed utilizing most other techniques.

In accordance with the invention, cyclin dependent kinase may either be measured directly or, alternatively, by measuring other parameters which are indicative of cyclin dependent kinase concentration. These other parameters may be parameters which vary with cyclin dependent kinase concentration, or even parameters which vary inversely with cyclin dependent kinase. For example, in some embodiments of the invention, parameters are measured which are related to either the formation or later metabolic fate of cyclin dependent kinase. For example, mRNA for cyclin dependent kinase could be measured, as could proteases involved in the degradation of cyclin dependent kinase. In one embodiment, tyrosylphosphorylation of cyclin dependent kinase is measured. The foregoing measurements are preferably performed by ELISA or immunohistochemical techniques utilizing antibodies to at least one cyclin dependent kinase, or to other antigens the concentration of which is indicative of cyclin dependent kinase concentration (e.g., some of the related parameters discussed above).

After measurements are taken, it is preferred but not required that measurements be compared to a

control which may be either a historical or concurrent control, standard curve, archival materials, or the like. However, for a given purpose, a user's own prior experience with the measurement, and with its implications may be sufficient for subjective evaluation of the measurement by the user. In some embodiments of the invention, "before" and "after" measurements are taken to determine the effect of an intervening regimen or of exposure to stimulus. In other embodiments, abnormal measurement levels based on historical or archival data or standard curves are determined. Depending upon the sensitivity desired, for example, a positive indication could be set at one, two or three standard deviations above the mean of a normal control. Those of skill in the art will recognize a wide range of uses for the present methods and kits, only a representative sample of which are discussed below.

For purposes of diagnostic evaluation of tissues, samples suspected of having undergone transformation to cancerous phenotype, e.g., breast, prostate, colon, lung, stomach or pancreas tissues, or lymphocytes, etc., may be subjected to the methods of the present invention wherein abnormal measurements of parameters indicative of cyclin dependent kinase concentration will represent a positive signal for transformation to cancerous phenotype or likelihood of transformation. The ability to determine likelihood of transformation is of particular value in biopsy, especially when a patient is to undergo surgery for removal of a tumor. The present invention provides an improved method of determining how radical such surgery should be, and how much tissue should be removed.

Other applications include the diagnostic evaluation of potential agents to induce cancer phenotype in any cell or tissues. Comparative testing could be done, for example, utilizing fish or other animals from waters polluted with certain pollutants (the same animals from cleaner water could be used as controls).

The invention also has research applications to laboratory animals, and to providing model *in vitro* systems for the potency of carcinogenic agents or potential antineoplastic agents.

It is also possible, for example, to test the potency of potential inhibitors of various biological responses. A cell's response to a mitogen can be measured in accordance with the present invention, and the response to a combination of mitogen and a test inhibitor (at increasing concentrations) can also be tested by the present invention. The decrease in proliferation induced by the mitogen at increasing concentrations of inhibitor can be shown by measuring cyclin dependent kinase concentration in accordance with the invention, thereby providing a test of the effectiveness of the inhibitor.

The test of the present invention could also be used to establish "no effect" thresholds for toxic effects of various compounds. The test of the invention can be utilized, for example, to determine a threshold concentration below which the test compound will not interfere, for example, with the function of liver cells tested in accordance with the invention.

The present invention is able to provide statistically significant results after a very short period of time of cell incubation with a test compound. In preferred embodiments, *in vivo* tests involved

administering a test compound to a animal and allowing about 24 hours before sampling tissue. In *in vitro* tests, 24-48 hours of cell exposure to a test compound is preferred.

In certain embodiments of an *in vitro* test for carcinogenicity of a test compound, cells are synchronized at operational G. by deprivation of growth factors. The test compound is administered to some dishes, serum to a positive control, and nothing to a negative control. After about 24-48 hours, cells are harvested and lysed, the lysate being subjected to measurement of a parameter indicative of cyclin dependent kinase concentration in accordance with the invention. In one embodiment of this in vitro test, the cells to be tested are lysed (they should be kept cold through the procedure). Preferably, they are kept on ice and their temperature does not exceed 2-4°C. Both sample and standards are then bound to the plate, after diluting with a sample dilution buffer, e.g. a sodium borate buffer at pH 10.5 (about 100mM). The protein concentration is preferably between 6 and 100 μg per ml of dilution buffer. In accordance with standard ELISA techniques, the above binding is preferably followed by blocking the remaining sites, adding the primary antibody (anti-PSTAIR by way of example only), adding the secondary antibody and color development (where the secondary antibody is detectable by color).

In a corresponding *in vivo* application, animal tissue is obtained about 24 hours after exposure to a test compound. The tissue is preferably slurried and then subjected to testing of a parameter indicative of cyclin dependent kinase concentration. One preferred embodiment proceeds like the *in vitro* test above.

Protein concentration for the *in vivo* test being preferably between 12.5 and 50 μg protein per ml of dilution buffer.

Preferred kits of the invention provide a lysate buffer for an *in vitro* test, or homogenization buffer for an *in vivo* test and a dilution buffer for both.

Immunohistochemical analysis of cells suspected of having transformed to a cancerous phenotype, or suspected of having increased susceptibility to transformation, may proceed in an analogous manner starting with a thin (e.g. 4-6 micron) sample immobilized on a slide that has preferably been microwaved for about 10 minutes. Positive and negative controls are preferably provided on the slide.

Naturally, it is preferred that the antibodies used are specific for the particular antigen being measured and that the antibody formulations are substantially free of contaminants and of other antibodies to avoid cross-reactivity. The antibodies may be, for example, anti- cyclin dependent kinase (when cyclin dependent kinase concentration is being measured directly). Preferred anti- cyclin dependent kinase includes but is not limited to anti-PSTAIR, and antibodies to cyclin dependent kinases having an apparent molecular weight between about 32 and 34 kD, especially 33 kD and 34 kD, when measured on polyacrylamide gel.

Possible substances that may be tested in accordance with the invention include peroxisome proliferators, estrogens, estrogen receptor, testosterone, testosterone receptor. The invention may also measure carcogenicity of compounds from the dioxin or PCB group.

As used herein, "cell samples" may include tissues that have the type of cells under discussion.

A method and assay to determine whether a test compound or sample is a nongenotoxic carcinogen, wherein the compound or sample to be tested is added to a cyclin dependent kinase (CDK) assay system is provided. The assay system can be inter alia a living organism, a cell culture or a cell lysate, as long as the assay system contains a cyclin dependent kinase (CDK). An increase in the tyrosylphosphorylation level of CDK (one indication of increased CDK concentration) indicates that the test compound is a nongenotoxic carcinogen, or that the test sample contains a nongenotoxic carcinogen.

This assay also detects nonmutagenic carcinogens and substances having a cell proliferation effect. The nongenotoxic carcinogens that can be identified through the assay include tumor promoters, chlorinated biphenyls, hormones, dioxins and peroxisome proliferators, among others. The assay system can be assembled in the form of a test kit for diagnostic and environmental testing.

The above assay could also be used to quantify the potency of a particular growth factor (peptide hormone). A peptide growth factor would be added to the assay system instead of a xenobiotic (foreign chemical) and otherwise the assay would proceed without modification.

The method and assay of the invention can also be used to determine the potential of a chemical as an antineoplastic agent by reversing the steps outlined above. Starting with a transformed cell or transformed cell lysate, a potential antineoplastic agent would be tested for the capacity of the chemical to put the cells

into the G_0 state. This capacity would be determined by quantifying the decrease in cyclin dependent kinase, e.g. by measuring tyrosylphosphorylation of the CDK. The only other modification necessary to convert the assay for nongenotoxic carcinogens to one for antineoplastic agents is to grow the neoplastic cells *in vitro* in a full serum complement (20% serum containing medium).

In Vivo Experiments

EXAMPLE 1

Enhanced tyrosylphosphorylation of $p34^{cdc2}$ kinase in an hepatic cytosol (S-9) preparation from C57BL/6J female mice 24 hours following administration of the nongenotoxic carcinogen 2,3,7,8-tetrachlordibenzo-p-dioxin.

Summary

$p34^{cdc2}$ is the serine/threonine kinase subunit of M-phase promoting factor (MPF) (29-31). The regulation of $p34^{cdc2}$ tyrosine phosphorylation status is considered the control mechanism for entry into G_1 from G_0, the START signal, and also from G_2 to M, the initiation of mitosis. It is demonstrated that a single dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin administered at 0.25, 0.5, 1, or 2 μg/kg to young, female mice increases the extent of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase compared to corn oil treated controls. These results indicate that the proliferative stimulus of the nongenotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin may be quantified as an increase in hepatic $p34^{cdc2}$ kinase tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase can serve to indicate the capacity of a dioxin-like chemical to function *in vivo* as a nongenotoxic carcinogen.

Materials and Methods

Chemicals: 2,3,7,8-tetrachlorodibenzao-p-dioxin (TCDD) is purchased from AccuStandard, Inc. (New Haven, CT).

Anti-phosphotyrosine monoclonal, anti-PSTAIR (CDK), and anti-$p34^{cdc2}$ kinase C-terminus polyclonal antibodies are obtained from UBI (Lake Placid, NY). The acronym PSTAIR is the abbreviation for the amino acid sequence used as the antigen for developing the anti-PSTAIR antibody. The two antibodies (PSTAIR and anti-C-terminus) recognize two different epitopes. At least nine CDKs have been described in the literature; these all have a common PSTAIR epitope. Therefore anti-PSTAIR would be expected to cross react with the entire complement of CDKs showing up in the 32 to 34 kD region. (Apparently some cyclins also cross react with the anti-PSTAIR antibody and this explains the banding at approximately 60 kD observed in some of the immunoblots with anti-PSTAIR.)

The antibody to the C-terminus region is more specific for $p34^{cdc2}$ kinase, since the C-terminus region is more variable than the highly conserved PSTAIR region. However, it is obviously not species-specific since it was generated against human cdc2 and it cross reacts with mouse, rat and dog $p34^{cdc2}$ kinase.

One or the other antibody is used depending upon the specificity desired in the experiments.

Bicinchoninic acid is obtained from Pierce (Rockford, IL). Molecular weight standards are supplied through BioRad (Melville, NY). All other chemicals are

purchased from Sigma (St. Louis, MO) and are of the highest purity available.

Animals and dosing: Four to six-wk old, female C57BL/6J mice are obtained from Harton Sprague Dawley (Indianapolis, IN). The mice are fed Prolab RMH 1000 (Agway, Cortland, NY) and receive tap water *ad libitum*. All mice are housed three per cage and maintained on a photoperiod of 12 h. Mice are killed 24 h following an intraperitoneal injection of TCDD in corn oil at 0, 0.25, 0.5, 1, or 2 μg/kg. Three mice are treated at each dose and the volume of the injections ranges from 0.1 to 0.2 mL per mouse. All preparation procedures are performed on pooled hepatic samples of the three mice per dose.

Preparation and -80°C storage of hepatic S-9 fractions is performed exactly as previously described in the scientific literature (32). This procedure involves killing the mouse by cervical dislocation, removing the liver and homogenizing the liver in three volumes of 0.15 M KCl. This hepatic homogenate is centrifuged at 9,000 x g for 20 min at 4°C. The resulting supernatant fraction, termed the S-9, is decanted into 1.5 mL plastic, conical tubes, frozen in a dry ice/ethanol bath and stored at -80°C until immunoprecipitation of phosphotyrosyl proteins can be performed.

Immunoprecipitation of tyrosine phosphorylated hepatic S-9 proteins with anti-phosphotyrosine monoclonal antibody: The hepatic S-9 is solubilized in immunoprecipitation buffer containing 20 mM Tris HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.15 U/mL aprotinin, and 1 mM sodium vanidate, centrifuged at 13,000 x g for 15

min at 4°C. The solubilized hepatic S-9 proteins are then incubated with anti-phosphotyrosine monoclonal antibody (5 μg/mL) at 4°C for 4 h or overnight. After the incubation period, add 25 μL of protein A-Sepharose for each 5 μg of antibody. One h later the immune complexes are collected by centrifugation at 13,000 x g, washed twice with immunoprecipitation buffer, solubilized in SDS gel sample buffer and heated at 100°C for 5 min in preparation of SDS PAGE and immunoblotting.

Gel electrophoresis and immunoblotting: SDS PAGE is carried out as described in the scientific literature (33) using 11% polyacrylamide gels with the modification that hepatic S-9 (100 μg protein/well) are subjected to heat treatment (100°C) for 3 min. The immunoblotting assay is performed as described by Towbin et al. (34), however a Milliblot SDE electroblot apparatus (Millipore, Bedford, MA), is used to transfer proteins from polyacrylamide gels to an Immobilon® membrane filter (Millipore,Bedford, MA). Complete transfers are accomplished in 25-30 min at 500 mA and are assessed by tracking pre-stained molecular weight standards on the membrane filter.

Membrane filters are blocked by incubating in TBS (Tris buffered saline) containing 5% commercial nonfat dry milk (any commercial brand is suitable) for 30 min at room temperature. The membranes are then washed in TBST (TBS with 0.05% Tween 20) and incubated for 2 h with anti-human CDK (PSTAIR) antibody (2 - 5 μg/mL) in TBST or anti-mouse cdc2 kinase (C-terminus) polyclonal antibody in TBST. The antibody reaction is visualized by incubating the membranes for 2 h at room temperature with alkaline phosphatase-conjugated anti-mouse IgG diluted

1:1000 in TBST and developed for 15 min. Molecular weights are determined by adding molecular weight standards (Bio Rad, Melville, NY) to reference lanes and staining the membrane filters with amido black 10B. The resulting immunoblots are scanned into TIFF-formatted files (MacIntosh®; Apple Computers, Cupertino, CA) with a Microtech 600GS scanner (Torrance, CA) and quantified using Scan Analysis (BIOSOFT, Cambridge, UK). Summary scans are then printed and peak heights are measured directly from the figure. One density unit (U) is defined as one mm of the resulting peak height.

Protein determination: Bicinchoninic acid is used for the spectrophotometric determination of protein concentration (35). Mix 100 μL of sample (standard or unknown) with 2 mL of working reagent in a test tube. Color development occurs by incubation at 37oC for 30 min. Absorbance is read at 562 nm. Working reagent is made by adding 100 volumes of Reagent A with 2 volumes Reagent B. Reagent A: is made by combining 1.0 g bicinchoninic acid (Pierce Chemical, Rockford, IL); 2.0 g $Na_2CO_3*H_2O$; 0.16 g NaOH; and 0.95 g $NaHCO_3$ with water to 100 mL and adjust the pH to 11.25 with 50% NaOH. Reagent B consists of 4.9 g $CuSO_4*5H_2O$ to 100 mL in double distilled H_2O.

Results:

The anti-phosphotyrosine immunoprecipitate of the murine hepatic S-9 is run on an 11% polyacrylamide gel as described above and immunoblotting is performed with the anti-PSTAIR monoclonal antibody. The resulting anti-PSTAIR immunoblot is depicted in Figure 4. Density scans of the immunoblot are presented in Figure 5 and the

quantification of these bands is presented in Figure 6. The bands in Figure 4 at 34 and 32 kDa immunoreactive with anti-PSTAIR have been identified as cyclin dependent kinases and at this time it is not known if they represent isoforms of a single $pp34^{cdc2}$ kinase or whether they are two separate cyclin dependent kinases (36). The large anti-PSTAIR immunoreactive band at approximately 60 kDa has been identified as a cyclin protein (37, 38).

The results demonstrate that the tyrosylphosphorylated CDK ($pp34^{cdc2}$) does not exist in measurable quantities in the hepatic S-9 of corn oil treated control mice. However, dosing of mice with TCDD enhanced the tyrosylphosphorylation of a p34 and p32 to a maximum at 0.5 μg TCDD/kg. At higher doses of TCDD the tyrosylphosphorylation of the kinase(s) becomes attenuated, perhaps due to overt toxicity of TCDD to the mice at these higher doses.

EXAMPLE 2

Enhanced tyrosylphosphorylation of $p34^{cdc2}$ kinase in an hepatic cytosol preparation (S-9) from young male rats 24 hours following administration of the nongenotoxic carcinogen pirnixic acid.

Summary

$p34^{cdc2}$ is the serine/threonine kinase subunit of M-phase promoting factor (MPF) (29-31). The regulation of $p34^{cdc2}$ tyrosine phosphorylation status is considered the control mechanism for entry into G_1 from G_0, the START signal, and also from G_2 to M, the initiation of mitosis. It is demonstrated that twice daily doses of 50 mg pirnixic acid/kg of body weight for 5 days to young male rats increases the extent of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase compared to corn oil treated

controls. These results indicate that the proliferative stimulus of the nongenotoxic carcinogen pirnixic acid may be quantified as an increase in hepatic $p34^{cdc2}$ kinase tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase can serve to indicate the capacity of chemicals that are termed peroxisome proliferators to function *in vivo* as a nongenotoxic carcinogen.

Materials and Methods

Chemicals: Pirnixic acid (CAS 50892-23-4 [4-chloro-6-(2,3-xylidino)-2-pyrimidiylthio] acetic acid) is purchased from ChemSyn Science Labs (Lenexa, KY). Anti-phosphotyrosine monoclonal, anti-PSTAIR (CDK), and anti-$p34^{cdc2}$ kinase C-terminus polyclonal antibodies are obtained from UBI (Lake Placid, NY). Bicinchoninic acid is obtained from Pierce (Rockford, IL). Molecular weight standards are supplied through BioRad (Melville, NY). Sensor Chips CM5, Surfactant P20, and amine coupling kit (EDC, NHS, and ethanolamine hydrochloride) were purchased from Pharmacia Biosensor AB. All other chemicals are purchased from Sigma (St. Louis, MO) and are of the highest purity available.

Animals and dosing: Eight-wk old male Sprague-Dawley rats are purchased from Charles River Laboratory (Charles River, MA) and housed four to a cage in polycarbonate cages (24 x 34 x 20 cm). Bedding consists of hardwood chips. Rats are allowed free access to tap water and fed Agway RMH 3000 (Cortland, NY) *ad libitum*. Photoperiod is maintained at 12 h of light and 12 h of darkness.

After a wk of acclimation to new surroundings, treatments are begun. The treatment consists of twice daily doses of the test compound administered by oral gavage. The pirnixic acid is dissolved in corn oil. Sham-treated animals are given an equal volume of plain corn oil. Doses are adjusted daily on the basis of weight. The volume of corn oil is generally on the order of 2 mL/ rat throughout the treatment period. The second dose is given between the h of 13:00-16:00, approximately 6 h after the first dose given between the h of 7:00 - 10:00. The pirnixic acid is administered for 5 days at a dose of 50 mg/kg twice a day.

On the day of sacrifice the rats are anesthetized with ethyl ether and decapitated. Livers are removed, weighed and homogenized using a Potter-Elvehjem® tissue grinder with 3 mL of ice-cold 0.15 M KCl per g of wet weight of liver. This material is pooled for each rat and spun in a high speed centrifuge (Beckman J2-MI, Beckman Instruments, Fullerton, CA) for 10 min at 9000 x g at 4°C. The supernatant liquid is decanted, distributed as aliquot and frozen at -90°C.

Gel electrophoresis and immunoblotting with anti-phosphotyrosine: These procedures are carried out essentially as described in Example 1 except that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Real-time quantification of total tyrosylphosphorylated $p34^{cdc2}$ kinase: Surface plasmon

resonance (SPR) is used for the real time quantification of $p34^{cdc2}$ kinase that exists in the tyrosylphosphorylated form. SPR is sensitive to changes in the optical properties of a medium close to a metal surface (39). SPR is suitable for macromolecular interaction studies at solid/liquid interfaces with the use of a carboxymethylated dextran hydrogel placed upon a thin layer of gold (39,40).

The detection system of a SPR monitor consists of a light source emanating both monochromatic and plane-polarized light, a glass prism, a thin metal film in contact with the base of the prism, and a photodetector. An evanescent field forms from the prism into the metal film when obliquely incident light on the base of the prism will exhibit total internal reflection for angles greater than the critical angle. This evanescent field can couple to an electromagnetic surface wave, a surface plasmon, at the metal/liquid interface. Coupling is achieved at a specific angle of incidence, the SPR angle (39).

The SPR angle is highly sensitive to changes in the reactive index of a thin layer adjacent to the metal surface which is sensed by the evanescent wave. Therefore, it is a volume close to the surface that is probed. For example, when a protein layer is adsorbed on the metal surface, keeping all other parameters constant, an increase in the surface concentration occurs and the SPR angle shifts to larger values (39). The magnitude of the shift, defined as the SPR response, depends on the mean refractive index change due to the adsorption in the probed volume (a function of mass).

Utilizing SPR, biospecific interaction analysis is performed in real time in conjunction with a flow

injection system and is as sensitive as other methods such as radiolabeling, fluorometry, and chemiluminescence. In short, biospecific interaction analysis is a sensitive, nonlabile way of examining interactions between macromolecules in real time (40-42).

SPR measurements are performed on a BIAcore unit manufactured by Pharmacia Biosensor AB (Uppsala, Sweden). Sensor Chips CM5, Surfactant P20, and amine coupling kit (EDC, NHS, and ethanolamine hydrochloride) were purchased from Pharmacia Biosensor AB.

Immobilization of PSTAIR and C-terminus antibodies via amine coupling was performed according to the general procedure recommended by the manufacturer. Briefly, the instrument was equilibrated with HBS buffer (10 mM HEPES, 150 mM NaCl, 0.05% surfactant P20, pH 7.4, and filtered with a 0.22 micron filter), then the following series of injections were made using the autosampler incorporated into the BIAcore unit:

(1) equal volumes of EDC (0.1 M in water) and NHS (0.1 M in water) were mixed and 35 μL injected to activate the carboxymethylated surface;

(2) ligand (35 μL, 50μg/mL in 10 mM sodium acetate pH 4.5) was then injected;

(3) the remaining NHS-esters on the surface were then deactivated with ethanolamine (35 μL, 1 M in water, pH 8.5);

(4) noncovalently bound material was then washed from the surface with hydrochloric acid (15 μL. 20 mM). Immobilizations were executed with a continuous flow of HBS at a flow rate of 5μL/min.

A typical sensorgram produced on immobilization of anti-cdc2 C-terminus is depicted in Figure 7. Time required for immobilization is approximately 30 min.

BIAcore assay for tyrosylphosphorylation of cycline dependent kinase (CDK)-Each binding/regeneration cycle is performed with a constant flow of HBS of 3 μL/min. Hepatic S-9 fractions of rats dosed with pirnixic acid or vehicle alone are diluted to a concentration of 1.5 mg protein/mL into exhausted FB-2 tissue culture supernatant liquid and incubated overnight at 4°C with anti-phosphotyrosine antibody. This equilibrated mixture (40 μL) is then injected over the immobilized PSTAIR and C-terminus antibodies and binding is recorded in RU. Binding is directly proportional to the amount of tyrosylphosphorylated protein interacting with the anti-PSTAIR or anti-C Terminus antibodies.

Interpretation of results

Immunoblots - For scans of immunoblots, a change in phosphotyrosylprotein content of $p34^{cdc2}$ kinase greater than 40 percent was considered biologically meaningful.

BIAcore assay- Research on the cell cycle has shown that the concentration of cdc2 kinase remains constant and that tyrosine phosphorylation can be utilized as a marker of cells that are preparing to enter the M phase of the cell cycle (43-48). Therefore, increased binding indicate increased tyrosylphosphorylation of cdc2 kinase, thus more cells are in the process of preparing to enter mitosis. Treatment effects from BIAcore analyses are considered significant when the instrument response of the treatment group is outside the upper bounds of the population 95 percent confidence interval ($t_{(5)(0.95)} = 2.015$ times the

standard deviation of the RU response of the control animals).

Results

Immunoblotting analysis - As seen in Figure 8, seven proteins exhibited an increased tyrosine phosphorylation in response to the administration of pirnixic acid. A 6.24-fold increase was noted in pp69, while the greatest relative difference in peak height was seen with a 13.16-fold increase in pp33. Five phosphotyrosylproteins also evidenced a decrease in quantity. These were pp84, pp61, pp43, pp34 and pp23. Figure 9 depicts the scanning results and Figure 10 shows the quantification of the CDK at 33 kDa. Results indicate that the administration of five, twice-daily doses of pirnixic acid (50 mg/kg each dose) produces enhanced tyrosylphosphorylation of the CDK compared to control animals, which exhibit no tyrosylphosphorylation of CDK at 33 kDa. Each group on the graph represents the average of two rats. Error bars in this figure represent the 10 percent coefficient of variation in the quantification of density.

BIAcore (SPR) - Hepatic S-9 samples from rats treated with pirnixic acid produced greater binding to both anti-PSTAIR or anti-C-terminus antibodies than hepatic S-9 samples from vehicle control rats (Figure 11). This increased binding exhibited by the hepatic S-9 of test animals is due to enhanced tyrosylphosphorylation of cdc2 kinase (CDK). Figure 12 is a summary bar graph depicting BIAcore® quantification of the interaction of tyrosylphosphorylated cyclin dependent kinases (CDK) with anti-CDK polyclonal antibodies (PSTAIR and C-terminus)

from control and pirnixic acid-treated rats. Error bars represent standard deviations of n = 6 (anti-PSTAIR) and n = 8 (anti-C-terminus) control rats. RU value for pirnixic acid-treated rats represents the mean of 2 animals. The treatment of rats with 50 mg pirnixic acid/kg twice a day for 5 days results in enhanced tyrosylphosphorylation of CDK ($p34^{cdc2}$ kinase) compared to control rats.

EXAMPLE 3

Enhanced tyrosylphosphorylation of $p34^{cdc2}$ kinase in an hepatic cytosol preparation (S-9) from young male rats 24 hours following administration of the nongenotoxic carcinogen diethylhexylphthalate.

Summary

$p34^{cdc2}$ is the serine/threonine kinase subunit of M-phase promoting factor (MPF) (29-31). The regulation of $p34^{cdc2}$ tyrosine phosphorylation status is considered the control mechanism for entry into G_1 from G_0, the START signal, and also from G_2 to M, the initiation of mitosis. It is demonstrated that twice daily doses of 500 mg diethylhexylphthalate/kg of body weight for 5 days to young, male rats increases the extent of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase compared to corn oil treated controls. These results indicate that the proliferative stimulus of the nongenotoxic carcinogen diethylhexylphthalate may be quantified as an increase in hepatic $p34^{cdc2}$ kinase tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase can serve to indicate the capacity of chemicals that are termed peroxisome

proliferators to function *in vivo* as a nongenotoxic carcinogen.

Materials and Methods

Chemicals: Diethylhexylphthalate (DEHP) [CAS 117-81-7] was purchased from Fluka Chemicals (Ronkonkoma, NY). Anti-phosphotyrosine monoclonal, anti-PSTAIR (CDK), and anti-p34^{cdc2} kinase C-terminus polyclonal antibodies were obtained from UBI (Lake Placid, NY). Bicinchoninic acid was obtained from Pierce (Rockford, IL). Molecular weight standards were supplied through BioRad (Melville, NY). All other chemicals were purchased from Sigma (St. Louis, MO) and were of the highest purity available.

Animals and dosing: Rats are purchased and handled as described in Example 2.

After a wk of acclimation to new surroundings, treatments are begun. The treatment consists of twice daily doses of DEHP administered by oral gavage. The DEHP is dissolved in corn oil. Sham-treated animals are given an equal volume of plain corn oil. Doses are adjusted daily on the basis of weight. The volume of corn oil is generally on the order of 2 mL/rat throughout the treatment period. The second dose is given between the h of 13:00-16:00, approximately 6 h after the first dose given between the h of 7:00 - 10:00. The DEHP is administered for 5 days at a dose of 500 mg/kg twice a day. Rats are anesthetized and livers are prepared as described in Example 2.

Gel electrophor sis and immunoblotting with anti-phosphotyrosine: These procedures are carried out

essentially as described in Example 1 except that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 2.

Real-time quantification of total tyrosylphosphorylated $p34^{cdc2}$ kinase and interpretation of the results: These procedures are performed as described in Example 2.

Results

Immunoblotting analysis - Six phosphotyrosylproteins are shown to increase with the administration of DEHP (Figures 13 and 14). The range of relative increase is 1.48 to 4.19-fold. A decrease in pp31 and pp28 is also observed. Figure 15 depicts the quantification of the results of the scanning densitometry. The cyclin dependent kinase (CDK) quantified from the anti-phosphotyrosine immunoblot is at 34 kDa. Results indicate that the administration of five, twice-daily doses of DEHP (500 mg/kg each dose) produces enhanced tyrosylphosphorylation of the CDK compared to control animals, which exhibit no tyrosylphosphorylation of CDK at 34 kDa. Each group on the graph represents the average of two rats. Error bars represent the 10 percent coefficient of variation in the quantification of density.

BIAcore (SPR) - Hepatic S-9 samples from rats treated with DEHP produce greater binding to both anti-

PSTAIR or anti-C-terminus antibodies than hepatic S-9 samples from vehicle control rats (Figure 16). This increase in binding by the hepatic S-9 of DEHP-treated animals is due to enhanced tyrosylphosphorylation of cdc2 kinase (CDK). Figure 16 is a summary bar graph depicting BIAcore® quantification of the interaction of tyrosylphosphorylated cyclin dependent kinases (CDK) with anti-CDK polyclonal antibodies (PSTAIR and C-terminus) from control and DEHP-treated rats. Error bars represent standard deviations of n = 6 (anti-PSTAIR) and n = 8 (anti-C-terminus) control rats. RU value for DEHP-treated rats represents the mean of 2 animals. The treatment of rats with 500 mg DEHP/kg twice a day for 5 days results in enhanced tyrosylphosphorylation of CDK ($p34^{cdc2}$ kinase) compared to control rats.

EXAMPLE 4

The genotoxic carcinogen diethylnitrosamine does not enhanced tyrosylphosphorylation of $p34^{cdc2}$ kinase in an hepatic cytosol preparation (S-9) from young male rats 24 hours following administration.

Summary

$p34^{cdc2}$ is the serine/threonine kinase subunit of M-phase promoting factor (MPF) (29-31). The regulation of $p34^{cdc2}$ tyrosine phosphorylation status is considered the control mechanism for entry into G_1 from G_0, the START signal, and also from G_2 to M, the initiation of mitosis. It is demonstrated that twice daily doses of 500 mg diethylnitrosamine/kg of body weight for 5 days to young, male rats did not affect the extent of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase compared to corn oil treated controls. These results indicate that the early *in vivo* effects of the genotoxic carcinogen diethylnitrosamine can not be quantified

through a change in hepatic $p34^{cdc2}$ kinase tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of hepatic $p34^{cdc2}$ kinase is specific for nongenotoxic carcinogens.

Materials and Methods

Chemicals: Diethylnitrosamine (DEN) [CAS 55-18-5] was purchased from Fluka Chemicals (Ronkonkoma, NY). Anti-phosphotyrosine monoclonal, anti-PSTAIR (CDK), and anti-$p34^{cdc2}$ kinase C-terminus polyclonal antibodies were obtained from UBI (Lake Placid, NY). Bicinchoninic acid was obtained from Pierce (Rockford, IL). Molecular weight standards were supplied through BioRad (Melville, NY). All other chemicals were purchased from Sigma (St. Louis, MO) and were of the highest purity available.

Animals and dosing: Animals are purchased and handled as described in Example 2. After a wk of acclimation to new surroundings, treatments are begun. The treatment consists of twice daily doses of DEN administered by oral gavage. The DEN is dissolved in corn oil. Sham-treated animals are given an equal volume of plain corn oil. Doses are adjusted daily on the basis of weight. The volume of corn oil is generally on the order of 2 mL/rat throughout the treatment period. The second dose is given between the h of 13:00-16:00, approximately 6 h after the first dose given between the h of 7:00 - 10:00. The DEN is administered for 5 days at a dose of 500 mg/kg twice a day. Rats are anesthetized and livers are prepared as described in Example 2.

Gel electrophoresis and immunoblotting with anti-phosphotyrosine: These procedures are carried out as described in Example 1 except that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Real-time quantification of total tyrosylphosphorylated $p34^{cdc2}$ kinase and interpretation of the results: These procedures are performed as described in Example 2.

Results

Immunoblotting analysis - Administration of DEN to young male rats did not produce any increases in phosphotyrosylproteins (Figures 17 and 18). A 61% decrease in pp22 is observed. Figure 19 is a bar graph depicting the quantification of the results of the scanning densitometry. The band quantified from the anti-phosphotyrosine immunoblot is at 34 kDa. Results indicate that the administration of five, twice-daily doses of DEN (500 mg/kg each dose) produces no enhanced tyrosylphosphorylation of the p34 compared to control animals. Each group on the graph represents the average of two rats. Error bars represent the 10 percent coefficient of variation in the quantification of density.

BIAcore (SPR) - Hepatic S-9 samples from rats treated with DEN produce no greater binding to anti-PSTAIR or anti-C-terminus antibodies than hepatic S-9 samples from vehicle control rats. Figure 20 is a

summary bar graph depicting BIAcore® quantification of the interaction of tyrosylphosphorylated cyclin dependent kinases (CDK) with anti-CDK polyclonal antibodies (PSTAIR and C-terminus) from control and DEN-treated rats. Error bars represent standard deviations of n = 6 (anti-PSTAIR) and n = 8 (anti-C Terminus) control rats. RU value for DEN-treated rats represents the mean of 2 animals. Results indicate that the treatment of rats with 500 mg DEN/kg twice a day for 5 days produces no enhanced tyrosylphosphorylation of CDK ($p34^{cdc2}$ kinase) compared to control rats.

EXAMPLE 5

Enhanced tyrosylphosphorylation of p34 in an hepatic cytosol preparation (S-9) from female Beagle dogs following administration of the nongenotoxic carcinogen Aroclor® polychlorinated biphenyls for eleven and one-half weeks

Summary

It is demonstrated that daily doses of 0.6, 0.8, 4-8, or 5-10 mg /kg of body weight for 11.5 weeks to 2-year old, female Beagle dogs enhances the tyrosine phosphorylation status of an hepatic p34 compared to corn oil treated controls. These results indicate that the early *in vivo* effects of the nongenotoxic carcinogen Aroclor® polychlorinated biphenyls can be quantified through a change in hepatic p34 tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of hepatic p34 is specific for nongenotoxic carcinogens.

Materials and Methods

Chemicals: Aroclor®1254 polychlorinated biphenyls (PCBs) is purchased from AccuStandard, Inc.

(New Haven, CT). Anti-phosphotyrosine monoclonal antibody is obtained from UBI (Lake Placid, NY). Bicinchoninic acid is obtained from Pierce (Rockford, IL). Molecular weight standards are supplied through BioRad (Melville, NY). All other chemicals were purchased from Sigma (St. Louis, MO) or stated suppliers and were of the highest purity available.

Animals and dosing: Five, purebred, 2-year old, female beagle dogs, obtained from Norwich Pharmaceutical (Norwich, NY), are used in this study. All dogs were fully vaccinated, dewormed and specific pathogen free (SPF) for at least 30 days prior to the initiation of the experiment. They are maintained indoors and individually housed according to Public Health service guidelines (NIH publication No. 85-23). At the beginning of the study the dogs weigh between 8.7 and 12.2 kg. Physical observations of the dogs are made daily during the 11.5-wk dosing period of the study.

Each dog is administered either corn oil (controls) or Aroclor® PCBS at 0.6, 0.8, 4 or 5 mg/kg-day for seven wk. From seven to 11.5 wk, the 4 mg/kg-day dose and the 5 mg/kg-day dose are increased to 8 and 10 mg/kg-day, respectively. The corn oil, as well as test material, is administered in a cube of agarose concealed in a small ball of canned dog food. After consumption of the meatball, the dogs are immediately fed their daily caloric requirement of canned food.

Dogs were sacrificed using 2mL/kg of Fatal Plus (Vortech Pharmaceutical Company, Dearborne, MI). Hepatic S-9 fractions were prepared as previously described in Example 1.

Gel electrophoresis and immunoblotting with anti-phosphotyrosine: These procedures are carried out as described in Example 1 with the exception that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Results

The daily administration of Aroclor® polychlorinated biphenyls for a period of 11.5 wk results in enhanced tyrosylphosphorylation of a protein migrating at 34 kDa at all doses compared to the control dog. Figure 21 depicts the anti-phosphotyrosine immunoblot of dog hepatic S-9 protein separated using 11% SDS-PAGE gels for control and Aroclor® polychlorinated biphenyls-treated dogs. Lanes 1,2,3,4, and 5 are control, 0.6, 0.8, 4-8, and 5-10 mg Aroclor®/kg-day, respectively. The scanning densitometry of a single band at p34 of the anti-phosphotyrosine immunoblot is presented in Figure 22. Quantification of the scanning densitometry of p34 is presented in Figure 23 as a bar graph. Each bar on the graph represents the single result of scanning an immunoblot produced from the hepatic S-9 of one dog. Error bars represent the 10 percent coefficient of variation in the quantification of density.

In Vitro Experiments

EXAMPLE 6

Enhanced tyrosylphosphorylation of p34/p33 (putative CDK) in 3T3 cell lysates 24 hours following exposure to the nongenotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Summary

It is demonstrated that exposure of 3T3 cells to 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin for 24 h in a low serum media enhances the tyrosine phosphorylation status of two cell lysate proteins, p34 and p33, compared to dimethylsulfoxide-treated controls. These results indicate that the early *in vitro* effects of the nongenotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin can be quantified through a change in cellular p34/p33 tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of p34/p33 is specific for nongenotoxic carcinogens.

Materials and Methods

Chemicals: 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is purchased from AccuStandard, Inc. (New Haven, CT). Anti-phosphotyrosine monoclonal antibody is obtained from UBI (Lake Placid, NY). Bicinchoninic acid is obtained from Pierce (Rockford, IL). Molecular weight standards are supplied through BioRad (Melville, NY). All other chemicals were purchased from Sigma (St. Louis, MO) or stated suppliers and were of the highest purity available.

Tissue culture cells, culture conditions and dosing: 3T3 cells (ATCC CCL-92) are purchased from American Type

Culture Collection (Bethesda, MD). These cells are maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco cat. #430-2100) supplemented with 10% Fetal bovine serum-heat inactivated (FBS-HI) (Intergen, Purchase, NY). For experimental purposes, the cells are plated in 100 mm x 20 mm tissue culture dishes containing 10 mL of the above maintenance medium. The plates are placed in an incubator set at 37°C, 5% CO_2, 95% humidity, until they reach confluence (contact inhibited). At this point all the plates are then washed 2x with 5 mL of Dulbecco's calcium- and magnesium-free phosphate buffered saline (CMF-PBS). Four plates are then fed 10 mL of DMEM + 10% FBS-HI and all the other plates are fed 10 mL of DMEM + 0.5% FBS-HI and incubated for 48 h in the above environmental conditions.

After the 48 h incubation period, the medium from the low-serum group (0.5% FBS-HI) was aseptically harvested and allocated into separate tubes containing 40 mL each (to provide 10 mL/plate for 4 plates per treatment). The following concentrations and reagents are added to the appropriate tubes (4 plates/treatment). Dimethyl sulfoxide (DMSO) is used as the diluent for TCDD.

10 mL of DMEM + 20% FBS-HI + 0.1% DMSO
(positive control)
10 mL of DMEM + 0.5% FBS-HI + 0.1% DMSO
10 mL of DMEM + 0.5% FBS-HI + 10 nM TCDD

All plates were returned to the incubator for 24 h at the environmental conditions listed above. After the 24 h incubation period, the cells are harvested using the harvesting procedure described.

Gel electrophoresis and immunoblotting with anti-phosphotyrosine: These procedures are carried out as described in Example 1 with the exception that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Results

Exposure of 3T3 cells to 10 nM TCDD for 24 h results in an increase in tyrosylphosphorylation of p34 and p33 of 67 and 32%, respectively, compared to the vehicle control. The anti-phosphotyrosine immunoblot of 3T3 cell lysate protein separated using an 11% SDS-PAGE gel for 3T3 cells exposed to 10 nM TCDD is presented in Figure 24. Results of scanning the control and TCDD-treated lanes are presented in Figure 25; bolded peaks indicate p34 and p33 tyrosylphosphophoproteins. In Figure 26 the putative cyclin dependent kinases (p34/p33) are quantified from the anti-phosphotyrosine immunoblot. Results of serum supplementation (c.f. immunoblot in Figure 24, scan results not depicted in Figure 25) indicate enhanced tyrosylphosphorylation of p34/p33. This result would be expected if the pp34/pp33 are cyclin dependent kinases, since the serum supplemented media provide growth factor that stimulate the cells to mitosis and this stimulus is mediated through the CDK.

EXAMPLE 7

Enhanced tyrosylphosphorylation of p34/p33 (putative CDK) in 3T3 cell lysates 24 hours following exposure to the tumor promotor 12-O-tetra-decanoylphorbol-13-acetate.

Summary

It is demonstrated that exposure of 3T3 cells to 12-O-tetra-decanoylphorbol-13-acetate for 24 h in a low-serum media enhances the tyrosine phosphorylation status of two cell lysate proteins, p34 and p33, compared to dimethylsulfoxide-treated controls. These results indicate that the early *in vitro* effects of the tumor promotor 12-O-tetra-decanoylphorbol-13-acetate can be quantified through a change in cellular p34/p33 tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of p34/p33 is specific to a mechanism relating to the process of nongenotoxic carcinogenesis.

Materials and Methods

Chemicals: 2-O-Tetra-decanoylphorbol-13-acetate (TPA) is purchased from ChemSyn Science Labs (Lenexa, KY). Anti-phosphotyrosine monoclonal antibody is obtained from UBI (Lake Placid, NY). Bicinchoninic acid is obtained from Pierce (Rockford, IL). Molecular weight standards are supplied through BioRad (Melville, NY). All other chemicals were purchased from Sigma (St. Louis, MO) or stated suppliers and were of the highest purity available.

Tissue culture cells, culture conditions and dosing: These procedures are performed as described in Example 6. The following concentrations and reagents are added to the appropriate tubes (4 plates/treatment). Dimethyl sulfoxide (DMSO) is used as the diluent for TPA.

10 mL of DMEM + 20% FBS-HI + 0.1% DMSO
10 mL of DMEM + 0.5% FBS-HI + 0.1% DMSO
10 mL of DMEM + 0.5% FBS-HI + 160 nM TPA

Gel electrophoresis and immunoblotting with anti-phosphotyrosine: These procedures are carried out as described in Example 1 except that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Results

Exposure of 3T3 cells to 160 nM TPA for 24 h results in an increase in tyrosylphosphorylation of p34 and p33 of 54 and 95%, respectively, compared to the vehicle control. The anti-phosphotyrosine immunoblot of 3T3 cell lysate protein separated using an 11% SDS-PAGE gel for 3T3 cells exposed to 10 nM TCDD is presented in Figure 27. Results of scanning the control and TCDD-treated lanes are presented in Figure 28; bolded peaks indicate p34 and p33 tyrosylphosphophoproteins. In Figure 29 the putative cyclin dependent kinases (p34/p33) are quantified from the anti-phosphotyrosine immunoblot. Results of serum supplementation (c.f. immunoblot in Figure 27, scan results not depicted in Figure 28)

indicate enhanced tyrosylphosphorylation of p34/p33. This result would be expected if the pp34/pp33 are cyclin dependent kinases, since the serum supplemented media provide growth factor that stimulate the cells to mitosis and this stimulus is be mediated through the CDK.

EXAMPLE 8

Enhanced tyrosylphosphorylation of p34/p33 in BNL CL.2 cell lysates 24 hours following exposure to the nongenotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Summary

It is demonstrated that exposure of BNL CL.2 cells to 0.1, 1, 10 or 100 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin for 24 h in a low serum media enhances the tyrosine phosphorylation status of two cell lysate proteins, p34 and p33, compared to dimethylsulfoxide-treated controls. These results indicate that the early *in vitro* effects of the nongenotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin can be quantified through a change in cellular p34/p33 tyrosylphosphorylation and therefore that stimulation of tyrosylphosphorylation of p34/p33 is specific for nongenotoxic carcinogens.

Materials and Methods

Chemicals: This section is as previously described in Example 6.

Tissue culture cells, culture conditions and dosing: BNL CL.2 cells (ATCC TIB73) are purchased from American Type Culture Collection (Bethesda, MD). These cells are representative of normal mouse hepatocytes.

All other procedures were performed as detailed in Example 6.

The following concentrations and reagents are added to the appropriate tubes (4 plates/treatment). Dimethyl sulfoxide (DMSO) is used as the diluent for TCDD.

10 mL of DMEM + 20% FBS-HI + 0.1% DMSO (positive control)
10 mL of DMEM + 0.5% FBS-HI + 0.1% DMSO
10 mL of DMEM + 0.5% FBS-HI + 0.1 nM TCDD
10 mL of DMEM + 0.5% FBS-HI + 1.0 nM TCDD
10 mL of DMEM + 0.5% FBS-HI + 10 nM TCDD
10 mL of DMEM + 0.5% FBS-HI + 100 nM TCDD

All plates were returned to the incubator for 24 h at the environmental conditions listed above. After the 24 h incubation period, the cells are harvested using the harvesting procedure described.

Gel electrophoresis and immunoblotting with anti-phosphotyrosine: These procedures are carried out as described in Example 1 except that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Results

Exposure of BNL CL2 cells to 0.1, 1, 10 or 100 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for 24 h results in a simialr increase in tyrosylphosphorylation of p34, averaging 180% of the vehicle control over all

test concentrations of TCDD. Twenty percent serum supplementation results in an increase of tyrosylphosphorylation of p34 of 229% of the vehicle control. Vehicle controls at 0.5% serum supplementation exhibit no tyrosylphosphorylation at p33, while TCDD exposure at the four concentrations enhances tyrosylphosphorylation of this putative CDK to 0.9, 2.0, 2,0 and 1.9 density units, respectively. The increases in tyrosylphosphorylation of p33 by TCDD are 3.4 times the p33 tyrosine phosphorylation produced by 20% serum supplementation. The anti-phosphotyrosine immunoblot of BNL CL.2 cell lysate protein separated using an 11% SDS-PAGE gel for BNL CL.2 cells exposed to the four concentrations of TCDD is presented in Figure 30. Results of scanning the control and TCDD-treated lanes are presented in Figure 31; the represented peaks are p34 and p33 tyrosylphosphosphoproteins. In Figure 32 the putative cyclin dependent kinases (p34/p33) are quantified from the anti-phosphotyrosine immunoblot.

EXAMPLE 9

Enhanced tyrosylphosphorylation of p34/p33 in BNL CL.2 cell lysates 24 hours following exposure to the nongenotoxic carcinogen pirnixic acid.

Summary

It is demonstrated that exposure of BNL CL.2 cells to 1, 10 or 100 nM pirnixic acid for 24 h in a low serum media enhances the tyrosine phosphorylation status of two cell lysate proteins, p34 and p33, compared to dimethylsulfoxide-treated controls. These results indicate that the early *in vitro* effects of the nongenotoxic carcinogen pirnixic acid can be quantified

through a change in cellular p34/p33 tyrosylphosphorylation and that stimulation of tyrosylphosphorylation of p34/p33 is specific for nongenotoxic carcinogens.

Materials and Methods

Chemicals: This section is as previously described in Example 7.

Tissue culture cells, culture conditions and dosing: BNL CL.2 cells (ATCC TIB73) are purchased from American Type Culture Collection (Bethesda, MD). These cells are representative of normal mouse hepatocytes. All other procedures were performed as detailed in Example 7.

The following concentrations and reagents are added to the appropriate tubes (4 plates/treatment). Dimethyl sulfoxide (DMSO) is used as the diluent for TCDD.

10 mL of DMEM + 20% FBS-HI + 0.1% DMSO (positive control)

10 mL of DMEM + 0.5% FBS-HI + 0.1% DMSO

10 mL of DMEM + 0.5% FBS-HI + 1 nM pirnixic acid

10 mL of DMEM + 0.5% FBS-HI + 10 nM pirnixic acid

10 mL of DMEM + 0.5% FBS-HI + 100 nM pirnixic acid

10 mL of DMEM + 0.5% FBS-HI + 1000 nM pirnixic acid

All plates were returned to the incubator for 24 h at the environmental conditions listed above. After the 24 h incubation period, the cells are harvested using the harvesting procedure described.

Gel electrophoresis and immunoblotting with anti-phosphotyrosine: These procedures are carried out as described in Example 1 except that anti-phosphotyrosine antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Results

Exposure of BNL CL2 cells to pirnixic acid for 24 h results in increases in tyrosylphosphorylation of p34 relative to the vehicle control for the 1 and 10 nM concentrations, 96 and 58% increases, respectively. At 100 nM pirnixic acid the tyrosylphosphorylation of p34 is similar to the vehicle control, while at 1000 nM tyrosine phosphorylation of p34 is depressed 60% from the vehicle control. Twenty percent serum supplementation results in an increase of tyrosylphosphorylation of p34 of 229%, relative to the vehicle control. The 5% serum supplementation control exhibits no tyrosylphosphorylation at p33, while pirnixic acid exposure enhances tyrosylphosphorylation of this putative CDK to 2.0, 2.5 and 0.5 density units, respectively, at the 1, 10, and 100 nM concentrations. The increases in tyrosylphosphorylation of p33 by pirnixic acid at 1 and

10 nM are roughly 4 times the p33 tyrosine phosphorylation produced by 20% serum supplementation.

The anti-phosphotyrosine immunoblot of BNL CL.2 cell lysate protein separated using an 11% SDS-PAGE gel for BNL CL.2 cells exposed to the four concentrations of pirnixic acid is presented in Figure 33. Results of scanning the control and TCDD-treated lanes are presented in Figure 34; the represented peaks are p34 and p33 tyrosylphosphophoproteins. In Figure 35 the putative cyclin dependent kinases (p34/p33) are quantified from the anti-phosphotyrosine immunoblot.

EXAMPLE 10

Use of a microtiter assay for the assessment of enhanced tyrosylphosphorylation of cyclin-dependent kinases (CDK) or $p34^{cdc2}$ kinase in hepatic, pulmonary and renal cytosol (S-9) preparations from C57BL/6J female mice administered 2,3,78-tetrachlorodibenzo-p-dioxin for 90 days

Summary

The regulation of the tyroslyphosphorylaltion status of the cytosolic cyclin dependent kinases (CDK) is considered the control mechanism for the entry into G_1 from G_0, the START signal, and also for the movement of the cell from G_2 to M, the initiation of mitosis. A microtiter kit is described that allows for the demonstration of enhanced tyrosylphosphorylation of hepatic CDK as well as $p34^{cdc2}$ kinase following the daily administration of 0.25, 0.5, 1 or 2 ng 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)/kg to young, female mice for 90 days. It is also demonstrated that the microtiter kit may be used to assay for enhanced tyrosylphosphorylation of CDK in extrahepatic tissues and thus allow for the identification of the most sensitive responding tissue.

Materials and Methods

Materials and Chemicals:

Immobilon 2 microtiter plates	Dynatech (Shantilly, VA)
Anti-PSTAIR polyclonal antibody	UBI (Lake Placid, NY)
Anti-C-terminus polyclonal antibody	UBI (Lake Placid, NY)
Anti-phosphotyrosine monoclonal antibody	UBI (Lake Placid, NY)
Peroxidase-labeled rabbit anti-primary antibody	BioRad (Melville, NY)
BSA (bovine serum albumin)	[Sigma #A-3350]
Triton X-100	[Sigma #X-100]
EGTA (ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid)	[Sigma #E-4378]
PMSF (phenylmethylsulfonyl fluoride)	[Sigma #P-7626]
Leupeptin	[Sigma #L-2884]
Soy bean trypsin inhibitor	[Sigma #T-9003]
N-Tosyl-L-phenylalanine chloromethyl ketone	[Sigma #T-4376]
Sodium fluoride	[Sigma #S-6521]
β-Glycerophosphate	[Sigma #G-6626]
Paranitrophenyl phosphate	[Sigma #104-0]
Sodium orthovanadate	[Sigma #S-6508]
DTT (dithiothreitol)	[Sigma #D-0632]
$MgCl_2$	
ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonicacid) diammonium salt)	[Sigma #A-1888]
H_2O_2 (hydrogen peroxide)	[Sigma #H-1009]
TRIS	[Sigma (St. Louis, MO)]
Na Carbonate	[Sigma (St. Louis, MO)]
2,3,7,8-tetrachlorodibenzo-p-dioxin	[AccuStandard, Inc. (New Haven, CT)]

Reagents:

A. *Sodium carbonate buffer*; 0.1M, pH 9.6

a) Mix 71.3 ml of 1M $NaHCO_3$ and 28 ml of 1M Na_2CO_3.

b) Add 800 ml ddH_2O.

c) Adjust pH to 9.6 and Qs to 1 l.

B. *10X Phosphate buffered saline*; 0.15M, pH 7.2

a) NaCl, 80.0 g/l.

b) KCl, 2.0 g/l.

c) Na_2HPO_4, 11.5 g/l.

d) NaH_2PO_4, 2.0 g/l.

C. *Blocking buffer*; PBS with 3% BSA

a) 1X PBS with 3 g BSA per 100 ml.

D. *Washing buffer*; PBS with 0.2% Triton X-100

a) 1X PBS with 0.2 ml of Triton X-100 per ml.

E. *Prep Buffer*; 25mM Tris-HCl, pH 8.0 with 10mM $MgCl_2$, 15mM EGTA, 0.1% Triton X-100, 0.1mM PMSF, 0.1mM Na fluoride, 60mM β-glycerophosphate, 15mM paranitrophenylphosphate, 0.1mM Na orthovanidate, 1μg/ml leupeptin, 10μg/ml soybean trypsin inhibitor, 1μg/ml aprotinin, and 10μg/ml tosyl phenylalanine.

F. *Assay buffer*; 50mM Tris-HCl, pH 7.4 with 10mM $MgCl_2$, 1mM DTT, and all inhibitors of phosphatases and proteases contained in *Prep buffer*.

G. *Citrate buffer*;

a) Add 9.6 g Citric acid (MW 192.12) to 950 ml ddH_2O.

b) Adjust pH to 4.0 with 5M NaOH and store at 4°C.

H. *ABTS stock solution*;

a) 0.5487 g ABTS to 25 ml with double distilled H_2O and store at 4°C.

I. *ABTS substrate*; 0.4mM ABTS

a) 0.05 ml ABTS

b) 0.02 ml diluted H_2O_2 (0.5M)

c) 5.0 ml citrate buffer

Animals and dosing: Four to six-wk old, female C57BL/6J mice are obtained from Harton Sprague Dawley (Indianapolis, IN). The mice are fed Prolab RMH 1000 (Agway, Cortland, NY) and receive tap water *ad libitum*. All mice are housed three per cage and maintained on a photoperiod of 12 h. Mice are administered TCDD in corn oil at 0, 0.25, 0.5, 1, or 2 ng/kg by oral gavage daily for a period of 90 days. Ten mice are treated at each dose and the volume of the dose is approximately 0.1 mL per mouse.

Procedure:

Plate preparation:

1. 100 µl of anti-PSTAIR or anti-C-terminus antibody at a concentration of 10 µg/mL in of 0.1M *Na carbonate buffer* pH 9.6 is added to the wells of a microtiter plate and incubated overnight at 4°C. These are the capture antibodies and will retain all CDK or $p34^{cdc2}$ kinase, respectively.
2. Wash plates 3x with *washing buffer* by filling the wells, allowing them to sit for two minutes, and inverting and shaking them. This step removes all
3. Block plates for two hours at room temp by filling the wells with *blocking buffer*. The plates can be washed 1x with *washing buffer* and stored for several weeks at 4°C.
4. Wash fresh plated 3x or stored plates 2x with *washing buffer* prior to use.

Sample preparation:

All preparation procedures are performed on individual or pooled hepatic, pulmonary or renal samples. Preparation and -80°C storage of tissue S-9 fractions is performed exactly as previously described in the scientific literature (32). This procedure involves killing the mouse by cervical dislocation, removing the liver, lung or kidney sample and homogenizing the tissue in three volumes of *Prep buffer*. This tissue homogenate is centrifuged at 9,000 x g for 20 min at 4°C. The resulting supernatant fraction, termed the S-9, is decanted into 1.5 mL plastic, conical tubes, frozen in a dry ice/ethanol bath and stored at -80°C until the microtiter assay can be performed.

Assay:

1. 200 µg of sample tissue protein is diluted in *Prep buffer* and mixed 1:1 with *blocking buffer*.
2. This is added to the wells of a prepared plate and incubated for 5 hr at 4°C with slow constant shaking.
3. Plates are washed 3x with *washing buffer* and 1x with *assay buffer*.
4. 200 µl of primary (anti-phosphotyrosine) antibody at a dilution of 1:1000 in blocking buffer is added to each well and incubated for 2 hr at 4°C.
5. Plates are washed 3x with washing buffer.
6. 200 µl of peroxidase-conjugated (anti-mouse) secondary antibody at a dilution of 1:3000 in blocking buffer is added to each well and incubated for 1 hr at 4°C.
7. Wash plates 3x with washing buffer.

8. Add 200 µl of ABTS solution and read once a minute for 10 min in kinetics mode (Biotek EL312) at 415 nm.

Interpretation of results

Microtiter assay - The anti-PSTAIR or anti-C-terminus antibody will, respectively, capture all CDK or $p34^{cdc2}$ kinase present in the tissue S-9 fraction in the microtiter well. The anti-phosphotyrosine antibody quantifies the extent of tyrosylphosphorylation of the total CDK or p34cdc2 kinase. This quantification represents the extent to which the cells from the sampled tissue have been signaled to exit the Go stage of the cell cycle (index of proliferative signaling) by exposure to the test chemical. The current state of knowledge in the role of the cyclin dependent kinases in controlling the cell cycle (43-48) does not allow for an absolute determination as to the extent of CDK tyrosylphosphorylation relating to the strength of the proliferative signal. The fact that molecules other than peptide-like growth factors have the ability to enhance the tyrosylphosphorylation status of the CDK has not been reported in the literature. Therefore, interpretation of the capacity of a test chemical to direct the cell toward mitosis relies on a comparison to a control group treated only with the vehicle. A test chemical is considered positive for the capacity to function as a nongenotoxic carcinogen when the extent of CDK or $p34^{cdc2}$ kinase tyrosylphophorylation is statistically greater ($p < 0.05$) than a concurrent control.

Results

Microtiter assay - As seen in Figure 36, the dosing of C57BL/6J female mice with 0, 0.25. 0.5, 1 or 2 ng

TCDD/kg-day (A, B, C and D, respectively) for 90 days results in enhanced tyrosylphosphorylation of hepatic CDK but not pulmonary or renal CDK. This identifies the target tissue for the cellular proliferative effects of TCDD as the liver. Maximal increase in tyrosylphoshorylation of hepatic CDK is observed at the 0.5 ng TCDD/kg-day dose regimen. Results for the tryosylphosphorylation of $p34^{cdc2}$ kinase are similar (Figure 37), although the absolute increase observed is lower. This is due to the fact that p34cdc2 kinase represents only one of several possible CDK in the cytosol that function to regulate cell replecation.

EXAMPLE 11

Use of a microtiter assay for the assessment of enhanced expression of cyclin-dependent kinases (CDK) or $p34^{cdc2}$ kinase in hepatic cytosol (S-9) preparations from young male rats 1, 2, or 3 days following the administration of the nongenotoxic carcinogen pirinixic acid (WY14,643)

Summary

This example demonstrates of the utility of the assay for the quantification of CDK response elicited by a test chemical *in vivo* following an exposure period of any length and a description of a kit to perform the assay.

It is observed that the administration of the nongenotoxic carcinogen pirinixic acid to young, male rats results in the enhanced expression of total cytosolic cyclin-dependent kinases (CDK). A microtiter kit is described that allows for the demonstration of enhanced expression of hepatic CDK as well as $p34^{cdc2}$ kinase following a single dose of 50 mg pirinixic acid.

Materials and Methods

Materials and Chemicals:

Immobilon 2 microtiter plates	Dynatech (Shantilly, VA)
Anti-C-terminus cdc2 polyclonal antibody	UBI (Lake Placid, NY)
Anti-PSTAIR	UBI (Lake Placid, NY)
Peroxidase-labeled rabbit anti-primary antibody	BioRad (Melville, NY)
BSA (bovine serum albumin)	[Sigma #A-3350]
Triton X-100	[Sigma #X-100]
EGTA (ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid)	[Sigma #E-4378]
PMSF (phenylmethylsulfonyl fluoride)	[Sigma #P-7626]
Leupeptin	[Sigma #L-2884]
Soy bean trypsin inhibitor	[Sigma #T-9003]
N-Tosyl-L-phenylalanine chloromethyl ketone	[Sigma #T-4376]
Sodium fluoride	[Sigma #S-6521]
β-Glycerophosphate	[Sigma #G-6626]
Paranitrophenyl phosphate	[Sigma #104-0]
Sodium orthovanadate	[Sigma #S-6508]
DTT (dithiothreitol)	[Sigma #D-0632]
$MgCl_2$	
ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt)	[Sigma #A-1888]
H_2O_2 (hydrogen peroxide)	[Sigma #H-1009]
TRIS	[Sigma (St. Louis, MO)]
Na Carbonate	[Sigma (St. Louis, MO)]
Pirinixic acid	[ChemSyn Labs (Lenexa, KY)]

Reagents:

A. *Sodium carbonate buffer*; 0.1M, pH 9.6

a) Mix 71.3 ml of 1M $NaHCO_3$ and 28 ml of 1M Na_2CO_3.

b) Add 800 ml ddH_2O.

c) Adjust pH to 9.6 and Qs to 1 l.

B. *10X Phosphate buffered saline*; 0.15M, pH 7.2

a) NaCl, 80.0 g/l.

b) KCl, 2.0 g/l.

c) Na_2HPO_4, 11.5 g/l.

d) NaH_2PO_4, 2.0 g/l.

C. *Blocking buffer*; PBS with 3% BSA

a) 1X PBS with 3 g BSA per 100 ml.

D. *Washing buffer*; PBS with 0.2% Triton X-100

a) 1X PBS with 0.2 ml of Triton X-100 per ml.

E. *Prep Buffer*; 25mM Tris-HCl, pH 8.0 with 10mM $MgCl_2$, 15mM EGTA, 0.1% Triton X-100, 0.1mM PMSF, 0.1mM Na fluoride, 60mM β-glycerophosphate, 15mM paranitrophenylphosphate, 0.1mM Na orthovanidate, 1μg/ml leupeptin, 10μg/ml soybean trypsin inhibitor, 1μg/ml aprotinin, and 10μg/ml tosyl phenylalanine.

F. *Assay buffer*; 50mM Tris-HCl, pH 7.4 with 10mM $MgCl_2$, 1mM DTT, and all inhibitors of phosphatases and proteases contained in *Prep buffer*.

G. *Citrate buffer*;

a) Add 9.6 g Citric acid (MW 192.12) to 950 ml ddH_2O.

b) Adjust pH to 4.0 with 5M NaOH and store at 4°C.

H. *ABTS stock solution*;

a) 0.5487 g ABTS to 25 ml with double distilled H_2O and store at 4°C.

I. *ABTS substrate*; 0.4mM ABTS

a) 0.05 ml ABTS

b) 0.02 ml diluted H_2O_2 (0.5M)

c) 5.0 ml citrate buffer

Animals, dosing and preparation of tissue S9: This procedure is performed as described in Example 2 except only a single 50 mg/kg dose of pirinixic acid is administered. Livers are removed from rats on postdosing days 1, 2 and 3.

Gel electrophoresis and immunoblotting with anti-cdc2 C-terminus: These procedures are carried out as described in Example 1 except that anti-cdc2 C-terminus is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Microtiter assay procedure:

Sample preparation:

All preparation procedures are performed on individual or pooled hepatic (tissue) samples. Preparation and -80°C storage of tissue S9 fractions is performed exactly as previously described in the scientific literature (32). This procedure involves killing the rat by cervical dislocation, removing and homogenizing the tissue in three volumes of *Prep buffer*. This tissue homogenate is centrifuged at 9,000 x g for 20 min at 4°C. The resulting supernatant fraction, termed the S9, is decanted into 1.5 ml plastic, conical tubes, frozen in a dry ice/ethanol bath and stored at -80°C until the microtiter assay can be performed.

Assay:

1. 50 µg of S9 tissue protein is diluted in *Prep buffer* and mixed 1:1 with *blocking buffer*.

2. This is added to the wells of a prepared plate and incubated for 5 hr at 4°C with slow constant shaking.

3. Plates are washed 3x with *washing buffer* and 1x with *assay buffer*.

4. 200 µl of primary (anti-cdc2 C-terminus) antibody at a dilution of 1:1000 in blocking buffer is added to each well and incubated for 2 hr at 4°C.

5. Plates are washed 3x with washing buffer.

6. 200 µl of peroxidase-conjugated (anti-mouse) secondary antibody at a dilution of 1:3000 in blocking buffer is added to each well and incubated for 1 hr at 4°C.

7. Wash plates 3x with washing buffer.

8. Add 200 µl of ABTS solution and read once a minute for 10 min in kinetics mode (Biotek EL312) at 415 nm.

Interpretation of results

Microtiter assay - Due to cross-reactivity with other, unidentified CDK, the anti-cdc2 C-terminus antibody will quantify the total CDK expression in the tissue. This quantification represents the extent to which the cells from the sampled tissue have been signaled to exit the G_0 stage of the cell cycle (index of proliferative signaling) by exposure to the test chemical. The current state of knowledge in the role of the cyclin dependent kinases in controlling the cell cycle (43-48) does not allow for an explanation as to the strength of the proliferative signal. The fact that molecules other than peptide-like growth factors have the ability to enhance the expression of the CDK has not been reported in the literature. Therefore, interpretation of the capacity of a test chemical to direct the cell toward replication relies on a comparison to a concurrent control group treated only with the vehicle used to administer the test chemical. A test chemical is considered positive for the capacity to function as a nongenotoxic carcinogen when the extent of CDK or $p34^{cdc2}$ kinase expression is statistically greater ($p < 0.05$) than a concurrent control.

Results

Immunoblotting with anti-cdc2 C-terminus - Figure 38 depicts the immunoblot of rat hepatic S9 protein separated using 10 to 11% SDS-PAGE gels for control (lanes 1 and 3) and WY14,643-treated rats (lanes 2 and 4). A single intensely-stained band was visible in the CDK region (32 to 35 kDa) in hepatic S9 samples obtained from rats 3 days after receiving a single dose of 50 mg WY14,643/kg.

Microtiter assay - As seen in Figure 39, the extent of CDK expression in the livers of young, male rats

receiving a single dose of 50 mg/kg of WY14,643 increases steadily during the 3-day postdosing observation period. CDK expression in control animals remains constant over the same 3-day period.

EXAMPLE 12

Enhanced expression of CDK in BNL CL.2 cell lysates 48 hours following exposure to the nongenotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Summary

This example demonstrates the utility of the assay for the quantification of CDK response elicited by a test chemical in vitro following an exposure period of 48 hours.

It is demonstrated that exposure of BNL CL.2 cells to 0.1, 1, or 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin for 48 hours in a low serum media enhances the expression of two cell lysate proteins, p34 and p33 immunoreactive with anti-cdc2 C-terminus antibody, compared to dimethylsulfoxide-treated controls. These results indicate that the early in vitro effects of the nongenotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin can be quantified through a change in celluar CDK expression and therefore that stimulation of CDK is specific for nongenotoxic carcinogens.

Materials and Methods

Chemicals: This section is as previously described in Example 6.

Tissue culture cells, culture conditions and dosing: BNL CL.2 cells (ATCC TIB73) are purchased from American Type Culture Collection (Bethesda, MD). These

cells are representative of normal mouse hepatocytes. All other procedures were performed as detailed in Example 6.

The following concentrations and reagents are added to the appropriate tubes (4 plates/treatment). Dimethyl sulfoxide (DMSO) is used as the diluent for TCDD.

10 mL of DMEM + 20% FBS-HI + 0.1% DMSO (positive control)

10 mL of DMEM + 0.5% FBS-HI + 0.1% DMSO

10 mL of DMEM + 0.5% FBS-HI + 0.1 nM TCDD

10 mL of DMEM + 0.5% FBS-HI + 1.0 nM TCDD

10 mL of DMEM + 0.5% FBS-HI + 10 nM TCDD

All plates were returned to the incubator for 48 h at the environmental conditions listed above. After the 48 h incubation period, the cells are harvested using the harvesting procedure described.

Gel electrophoresis and immunoblotting with anti-cdc2 C-terminus: These procedures are carried out as described in Example 1 except that anti-cdc2 C-terminus antibody is used in place of anti-PSTAIR antibody.

Protein determination: This procedure is performed as described in Example 1.

Results

Exposure of BNL CL2 cells to 0.1, 1, or 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for 48 h results in an increase in expression of anti-cdc2 C-terminus immunoreactive proteins p34 and p33 compared to the serum deprived DMSO control (Figure 40, lanes 8, 9 and 10 compared to lane 6). CDK protein expression at 10 nM TCDD was similar to that observed with serum stimulation (lane 10 compared to lane 7).

EXAMPLE 13

Testing Chemical Compounds or
Test Samples for Nongenotoxic Carcinogens

The assays systems and methods disclosed in Examples 1-12 can be used to test chemical compounds, human and animal serum, air, water, and soil environmental samples for the presence of nongenotoxic carcinogens.

The above reagents, including antibodies, with or without aliquots of the cell lines described in the Examples may be packaged in the form of kits for the testing of suspected nongenotoxic carcinogens. Equivalent reagents, antibodies or cell lines may be substituted for the ones described in the Examples. In one preferred embodiment, a panel of three cell lines are included in the test kits. The three cell lines are a murine cell line, a rat cell line and a human cell line. Cell lines which are suitable for this purpose include murine BNL-CL.2 cells, a primary rat hepatic cell line developed by Paracelsian, Inc., PRLN-RH1, and a human hepatic cell line such as Hep G2 (ATCC: HB-8065).

Tissue samples, cells, and cell lysates from an individual person or animal can be substituted for the cell lines described, when testing for an individual's sensitivity to nongenotoxic carcinogens. Only reagents and antibodies would therefore be packaged in kits to test individual susceptibility.

Although the present invention has been described in relation to particular embodiments thereof, many other variations and modifications and other uses will become apparent to those skilled in the art. It is preferred, therefore, that the present invention be limited not by the specific disclosure herein, but only by the appended claims.

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WHAT IS CLAIMED IS:

1. A method for determining growth propensity for a tissue sample or cell line, said method comprising measuring a parameter that is indicative of concentration, in said sample or cell line, of at least one cyclin dependent kinase, and correlating said growth propensity to said measurement.

2. The method of claim 1, wherein said measurement is a direct measurement of the concentration of cyclin dependent kinase.

3. The method of claim 1, wherein said parameter is the concentration of $p34^{cdc2}$ or of a cyclin dependent kinase detectable at an apparent molecular weight between 32 and 34 kDa on a polyacrylamide gel.

4. The method of claim 1, wherein said parameter is the level of tyrosylphosphorylation of cyclin dependent kinase.

5. A diagnostic method for determining whether a tissue or cell sample has undergone transformation to a cancerous phenotype, said method comprising measuring a parameter indicative of concentration, in said tissue or cell sample, of at least one cyclin dependent kinase, and correlating said measurement to likelihood of transformation.

6. The method of claim 5, wherein said measurement is a direct measurement of the concentration of cyclin dependent kinase.

7. The method of claim 5, wherein said parameter is the concentration of $p34^{cdc2}$ or of a cyclin dependent kinase detectable at an apparent molecular weight between 32 and 34 kDa on a polyacrylamide gel.

8. The method of claim 5, wherein said parameter is the level of tyrosylphosphorylation of cyclin dependent kinase.

9. A diagnostic method for determining a likelihood that a tissue or cell sample will undergo transformation to a cancerous phenotype, said method comprising measuring a parameter that is indicative of concentration, in said tissue or cell sample, of at least one cyclin dependent kinase, and correlating said likelihood to said measurement.

10. The method of claim 9, wherein said measurement is a direct measurement of the concentration of cyclin dependent kinase.

11. The method of claim 9, wherein said parameter is the concentration of $p34^{cdc2}$ or of a cyclin dependent kinase detectable at an apparent molecular weight between 32 and 34 kDa on a polyacrylamide gel.

12. The method of claim 9, wherein said parameter is the level of tyrosylphosphorylation of cyclin dependent kinase.

13. A method of measuring carcinogenicity of a test substance comprising contacting said test substance with cells or tissue capable of expressing cyclin dependent

kinase and thereafter measuring a parameter indicative of concentration, in said cells or tissue, of at least one cyclin dependent kinase, and correlating said carcinogenicity with said measurement.

14. The method of claim 13, wherein said measurement is a direct measurement of the concentration of cyclin dependent kinase.

15. The method of claim 13, wherein said substance is contacted with an assay system selected from the group consisting of an animal, a cell culture, cell lines, or a panel of tissue.

16. The method of claim 13, wherein said test substance is selected from the group consisting of polychlorinated biphenyls, hormones and peroxisome proliferators.

17. The method of claim 13, wherein said parameter is the level of an mRNA coding for a cyclin dependent kinase.

18. The method of claim 13, wherein said test substance is nongenotoxic.

19. The method of claim 13, wherein said test substance is nonmutagenic.

20. The method of claim 13, wherein said parameter is the concentration of $p34^{cdc2}$.

21. The method of claim 13, wherein said parameter is the concentration of a cyclin dependent kinase detectable at an apparent molecular weight between 32 and 34 kDa on a polyacrylamide gel.

22. The method of claim 13, wherein said parameter is the level of tyrosylphosphorylation of cyclin dependent kinase.

23. The method of claim 13, wherein said measurement is performed on a cell lysate selected from the group consisting of 3T3 and BNL-CL.2.

24. A method of measuring effectiveness of a putative antineoplastic agent comprising the steps of:

(A) providing a sample of transformed cells;

(B) contacting said transformed cells with said putative antineoplastic agent;

(C) measuring a parameter indicative of concentration, in said cells, of at least one cyclin dependent kinase; and

(D) determining whether, or to what extent, said measurement indicates a decrease in cyclin dependent kinase following step (B).

25. The method of claim 24, wherein said parameter is measured both before and after step B, and said antineoplastic agent is evaluated by comparing the measurements taken before step (B) with measurements taken after step (B).

26. A kit for measuring cyclin dependent kinase concentration in human or animal cell lysates, said kit

including antibodies to an antigen whose concentration is indicative of cyclin dependent kinase concentration in said lysates.

27. The kit of claim 26, further including means for producing a standard curve from a standard having known cyclin dependent kinase content, or an historical standard curve.

28. The kit of claim 26, further comprising at least one inhibitor selected from the group consisting of a phosphate inhibitor and a protease inhibitor.

29. The kit of claim 26, wherein said antibodies are anti-cyclin dependent kinase antibodies.

30. The kit of claim 29, where an anti CDK antibody is selected from the group consisting of anti-C-terminis cdc2-polyclonal antibodies and anti-PSTAIR antibodies.

31. The kit of claim 26, further comprising:

(A) a lysate buffer;

(B) a means for receiving lysate and antibodies such that cyclin dependent kinase in said lysate may bind said antibodies;

(C) labelled secondary antibodies; and

(D) a means of detecting and quantifying said secondary antibodies.

32. A kit for measuring cyclin dependent kinase concentration in human or animal tissue or extracts, said kit including antibodies to an antigen whose concentration

is indicative of cyclin dependent kinase concentration in said tissues or extracts.

33. The kit of claim 32, wherein said kit further includes an historical standard curve or a means for producing a standard curve from a standard having known cycle dependent kinase content.

34. The kit of claim 32, further comprising at least one inhibitor selected from the group consisting of a phosphate inhibitor and a protease inhibitor.

35. The kit of claim 32, wherein said antibodies are anti-cyclin dependent kinase antibodies.

36. The kit of claim 35, wherein an anti CDK antibody is selected from the group consisting of anti-C-terminis cdc2 polyclonal antibody and anti-PSTAIR antibodies.

37. The kit of claim 36, further comprising:

(A) a homogenization buffer;

(B) a means for receiving a homogenate of said tissue and for receiving said antibodies such that cyclin dependent kinase in said homogenate may bind said antibodies;

(C) labelled secondary antibodies; and

(D) a means of detecting and quantifying said secondary antibodies.

38. An immunohistochemistry kit for determining whether cells or tissues have undergone transformation to a cancerous phenotype or are likely to undergo such transformation, said kit comprising a slide for receiving

a thin tissue slice containing said cells and further comprising an antibody to an antigen whose concentration is indicative of concentration of at least one cyclin dependent kinase in said cell or tissue sample.

39. The kit of claim 38, wherein said slide also includes, as separate slices, positive and negative control tissue.

40. The kit of claim 38, wherein said kit further includes an historical standard curve or means for producing a standard curve from a standard having known CDK content.

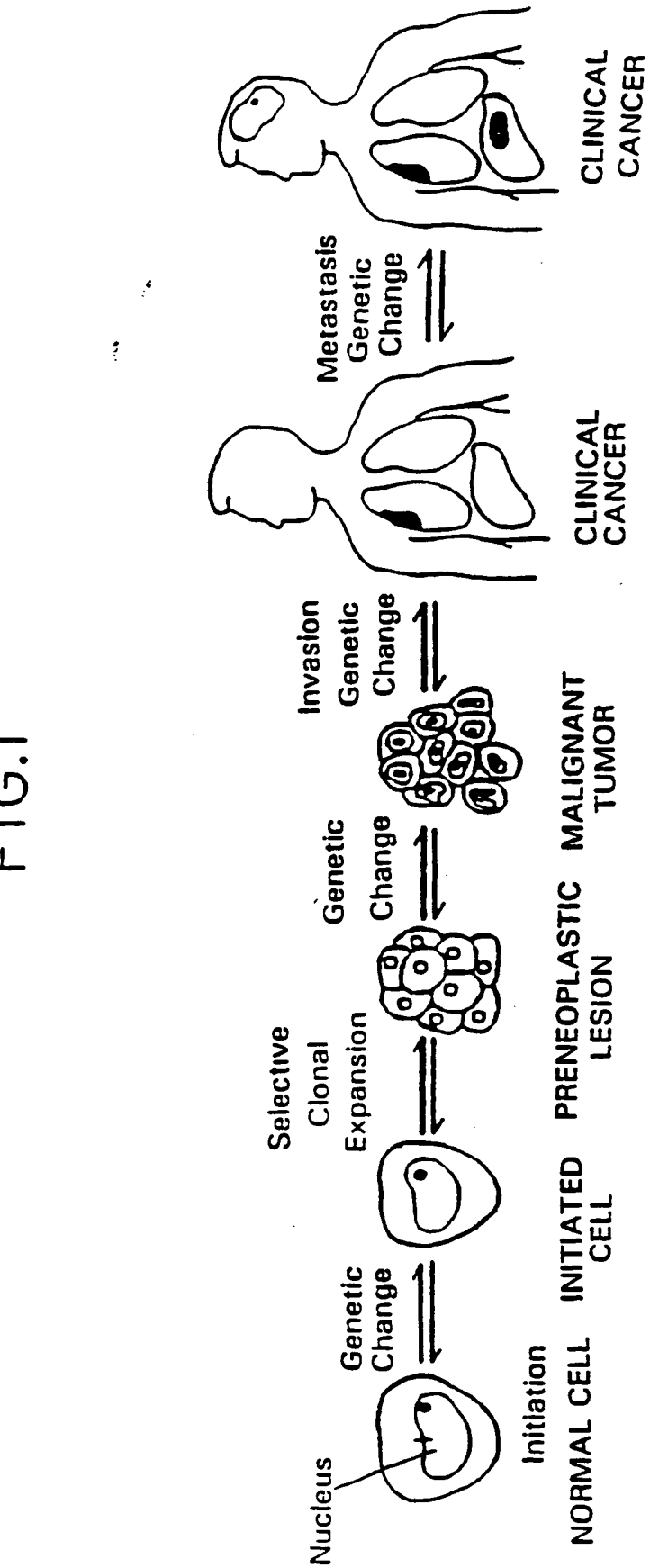
41. The kit of claim 38, further comprising at least one inhibitor selected from the group consisting of a phosphate inhibitor and a protease inhibitor.

42. The kit of claim 38, wherein the kit includes anti CDK antibody.

43. A method for determining efficacy of a regimen for reducing or enhancing cell growth, said method comprising the steps of measuring a parameter indicative of concentration levels of at least one cyclin dependent kinase following treatment of those cells with said regimen and correlating cyclin dependent kinase concentration with said efficacy.

44. The method of claim 43, wherein cyclin dependent kinase concentration is measured before and after beginning said regimen and said determination comprises comparing the measurement taken before with the measurement taken after.

FIG.1


Nucleus
Initiation
NORMAL CELL
Genetic Change
INITIATED CELL
Selective Clonal Expansion
Genetic Change
PRENEOPLASTIC LESION
Invasion Genetic Change
MALIGNANT TUMOR
Metastasis Genetic Change
CLINICAL CANCER
CLINICAL CANCER

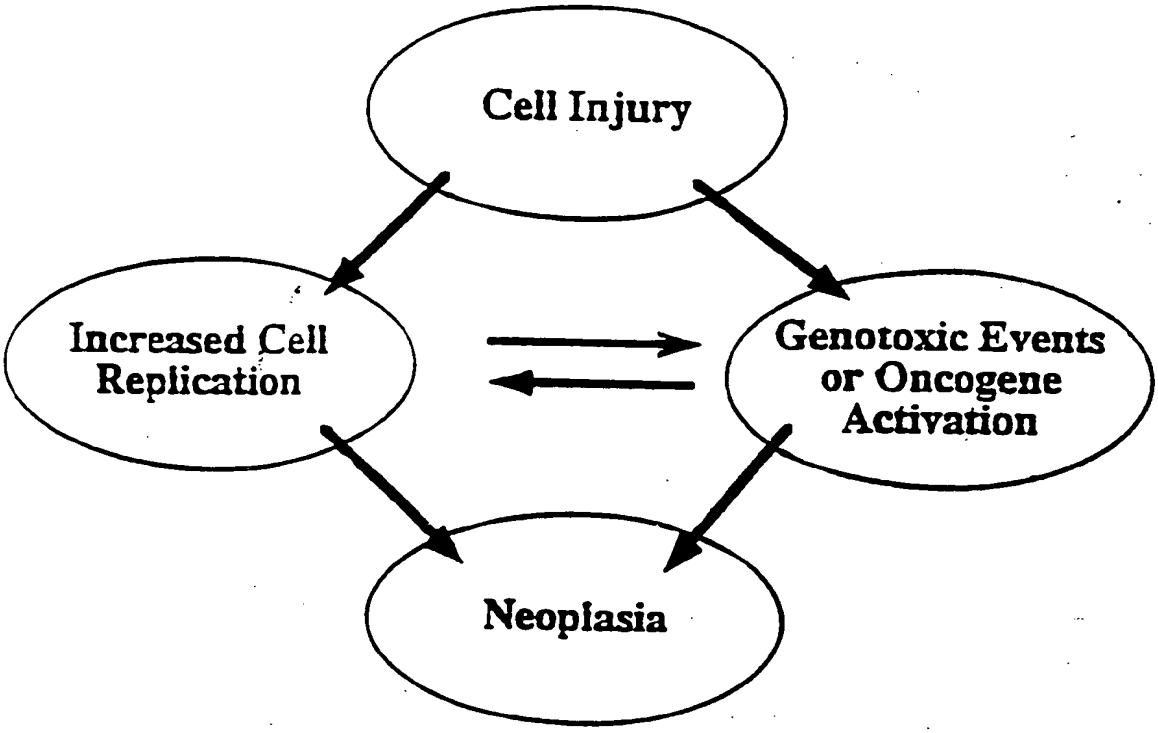

Cell Injury
Increased Cell Replication
Genotoxic Events or Oncogene Activation
Neoplasia

FIG. 2

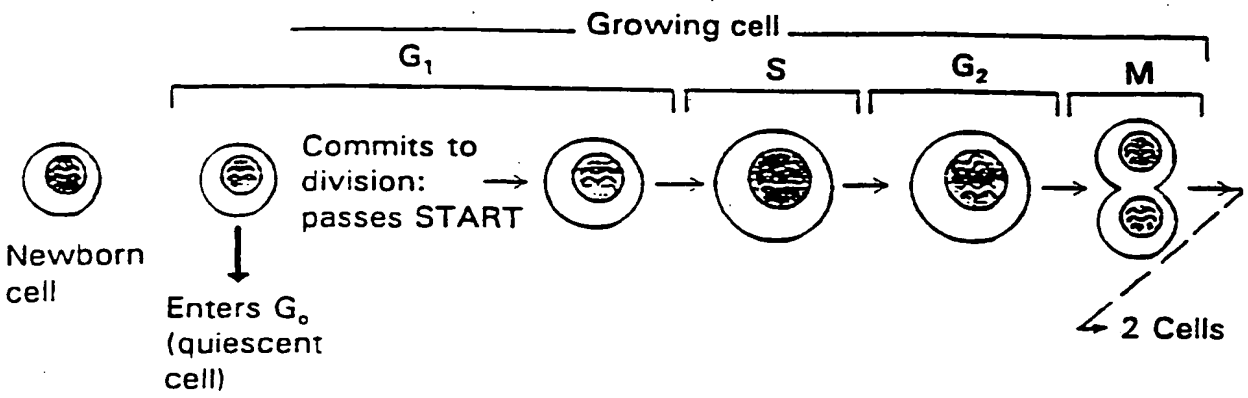

Growing cell
G1
S
G2
M
Commits to division: passes START
Newborn cell
Enters Go (quiescent cell)
2 Cells

FIG. 3

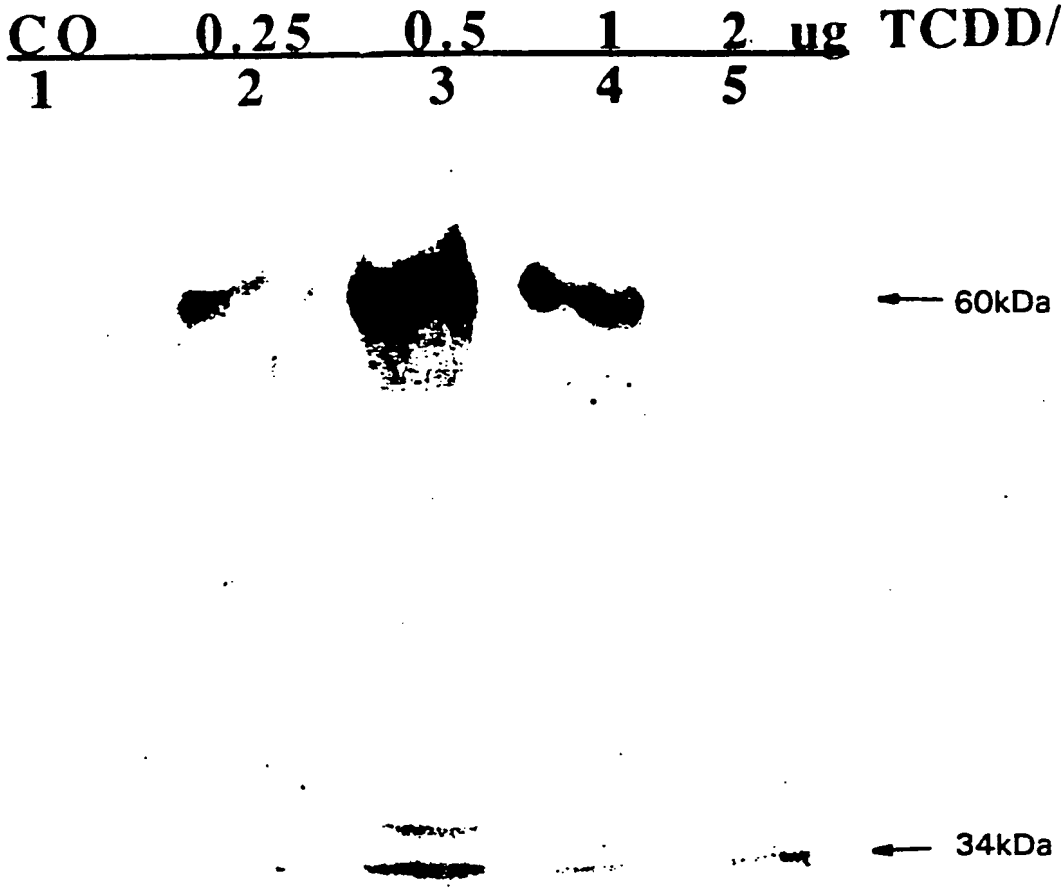


FIG. 4

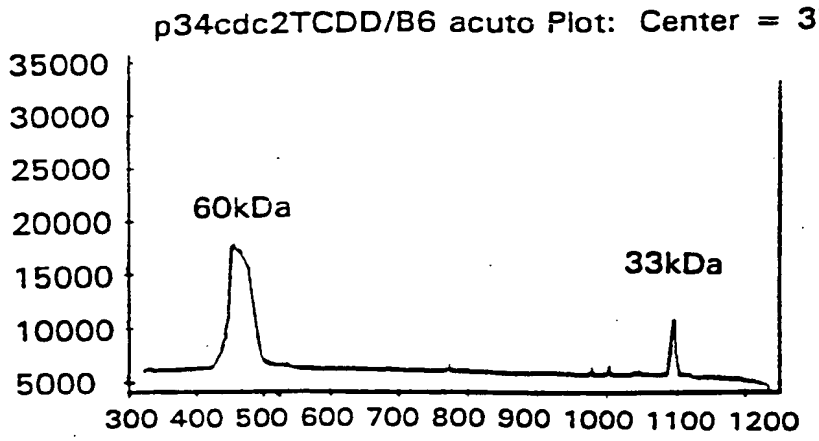


FIG. 5(a)

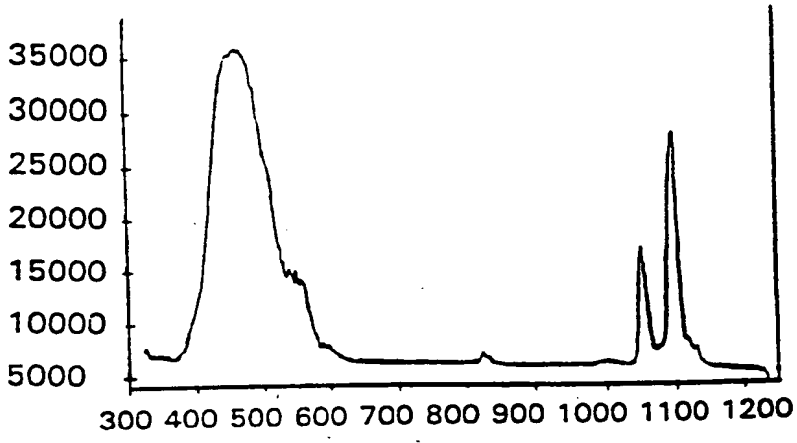


FIG. 5(b)

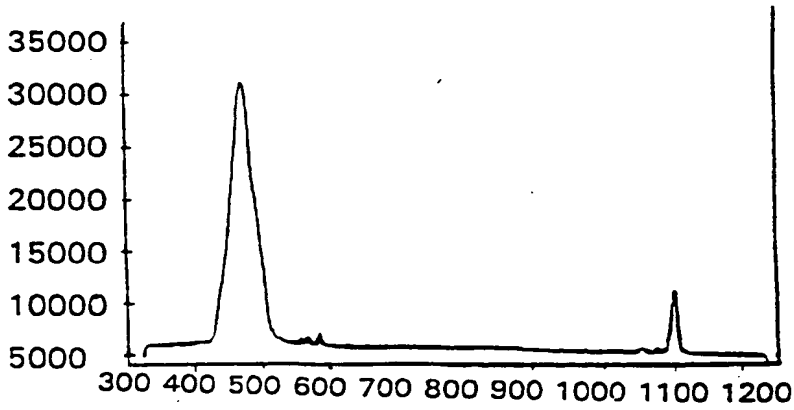


FIG. 5(c)

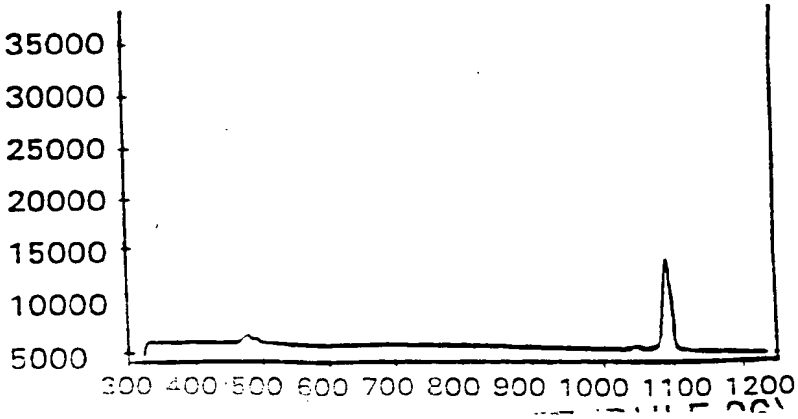


FIG. 5(d)

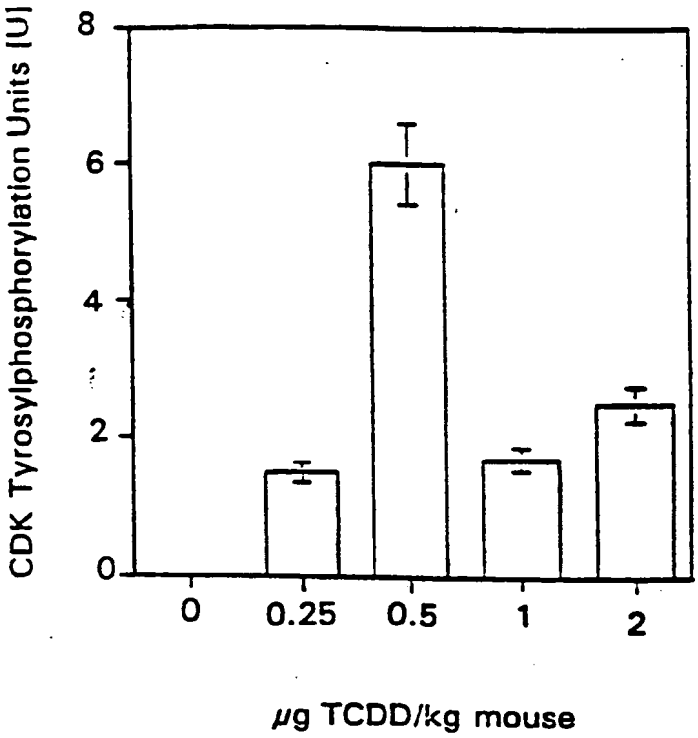

CDK Tyrosylphosphorylation Units (U)
8
6
4
2
0
0
0.25
0.5
1
2
µg TCDD/kg mouse

FIG. 6

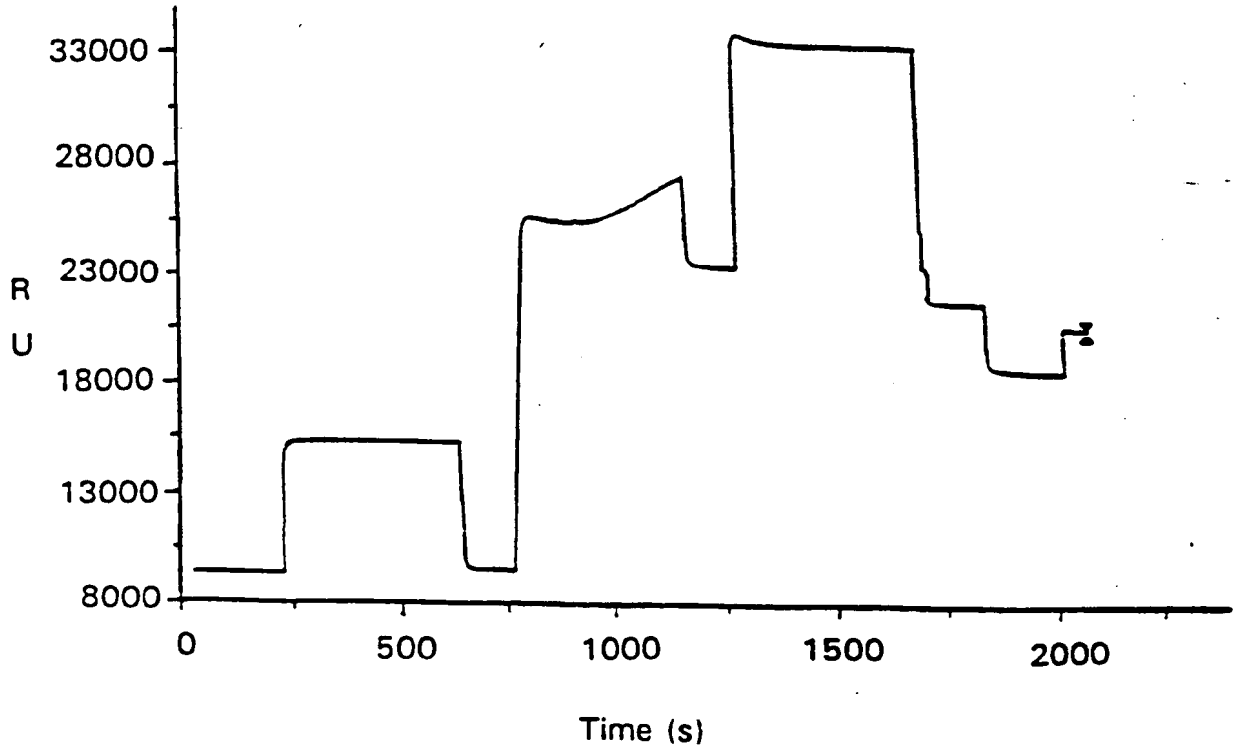

33000
28000
23000
18000
13000
8000
R
U
0
500
1000
1500
2000
Time (s)

FIG. 7

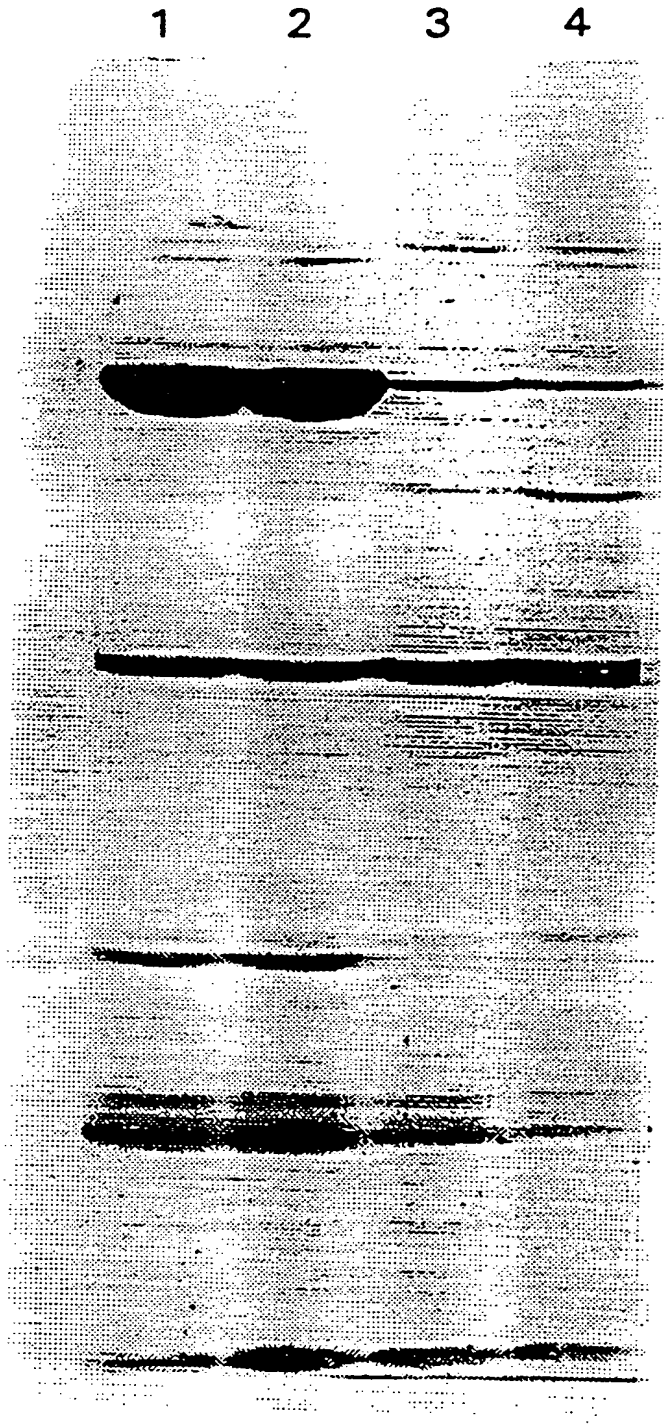


FIG. 8

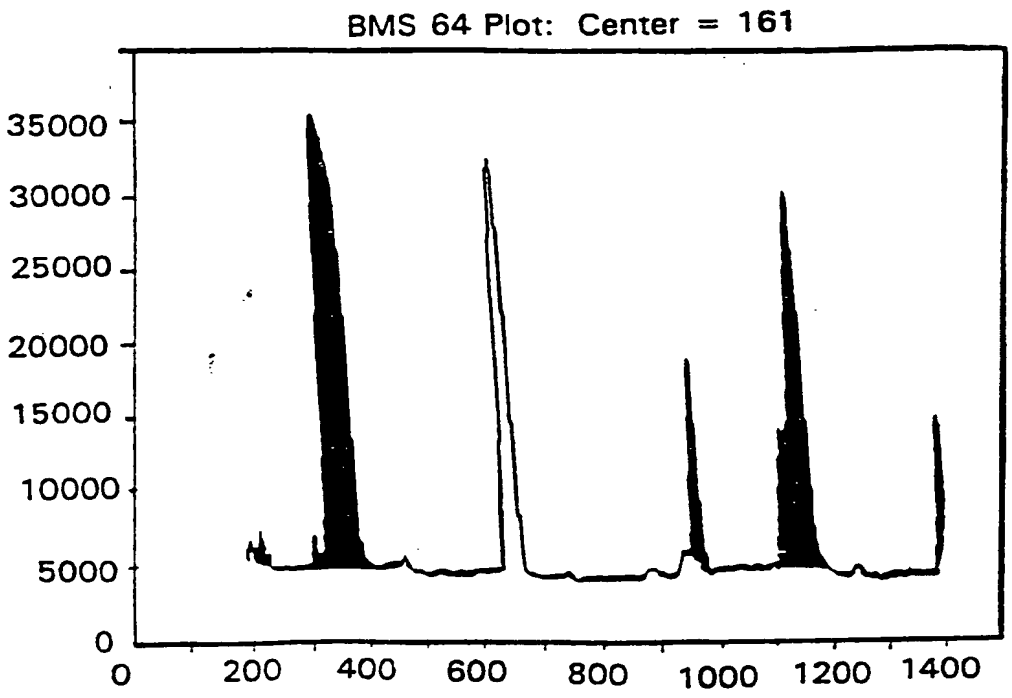

BMS 64 Plot: Center = 161
35000
30000
25000
20000
15000
10000
5000
0
0
200
400
600
800
1000
1200
1400

FIG.9(a)

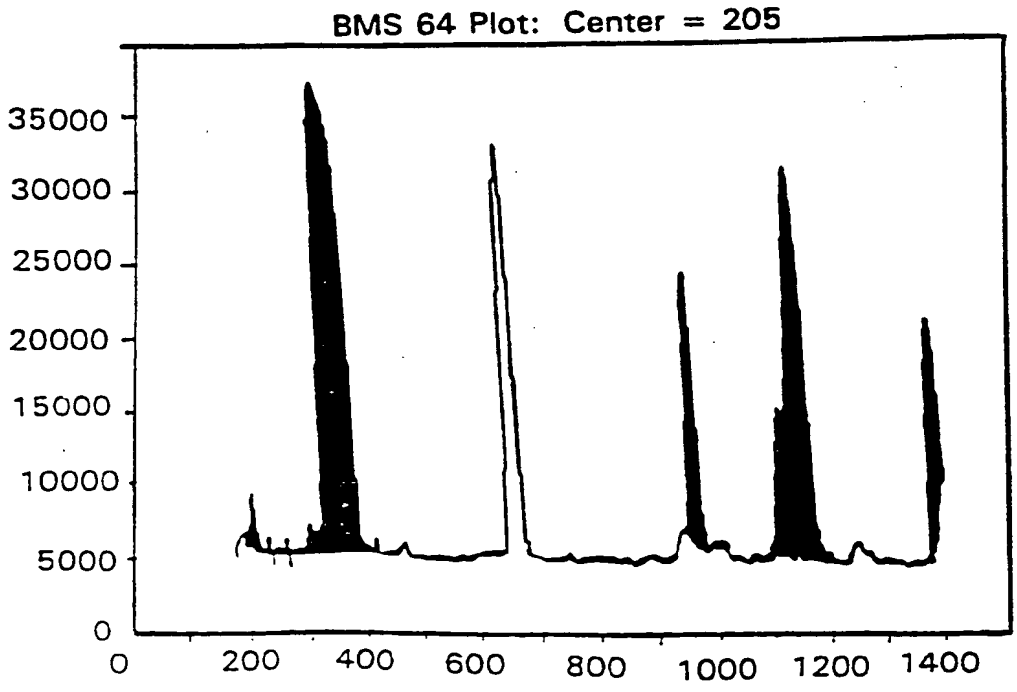

BMS 64 Plot: Center = 205
35000
30000
25000
20000
15000
10000
5000
0
0
200
400
600
800
1000
1200
1400

FIG.9(b)

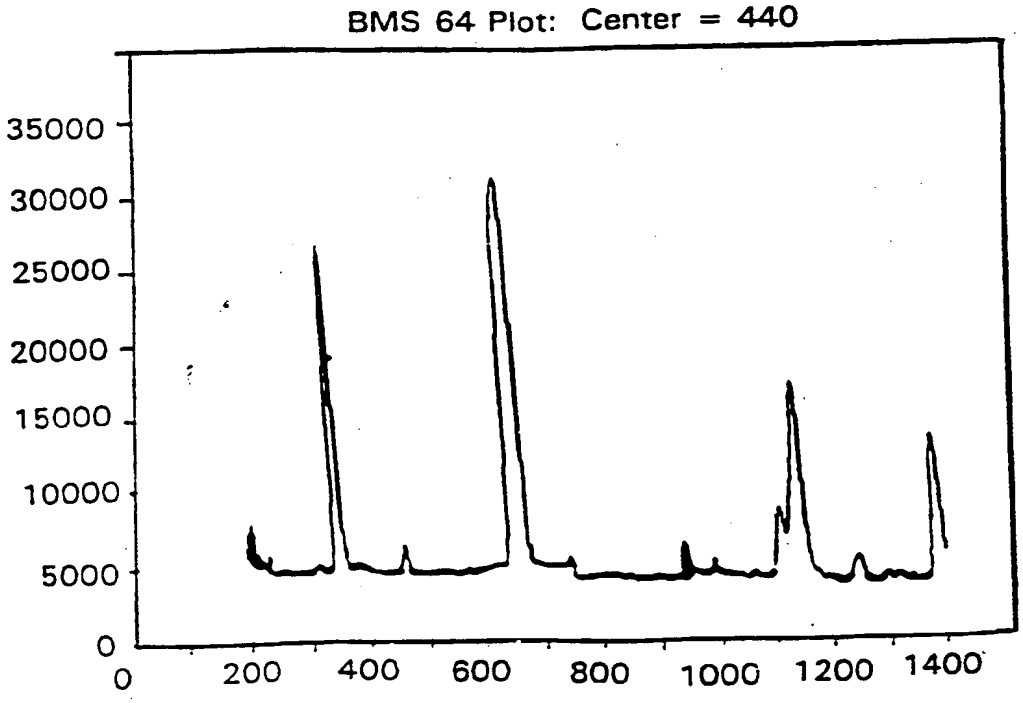

BMS 64 Plot: Center = 440
35000
30000
25000
20000
15000
10000
5000
0
0
200
400
600
800
1000
1200
1400

FIG.9(c)

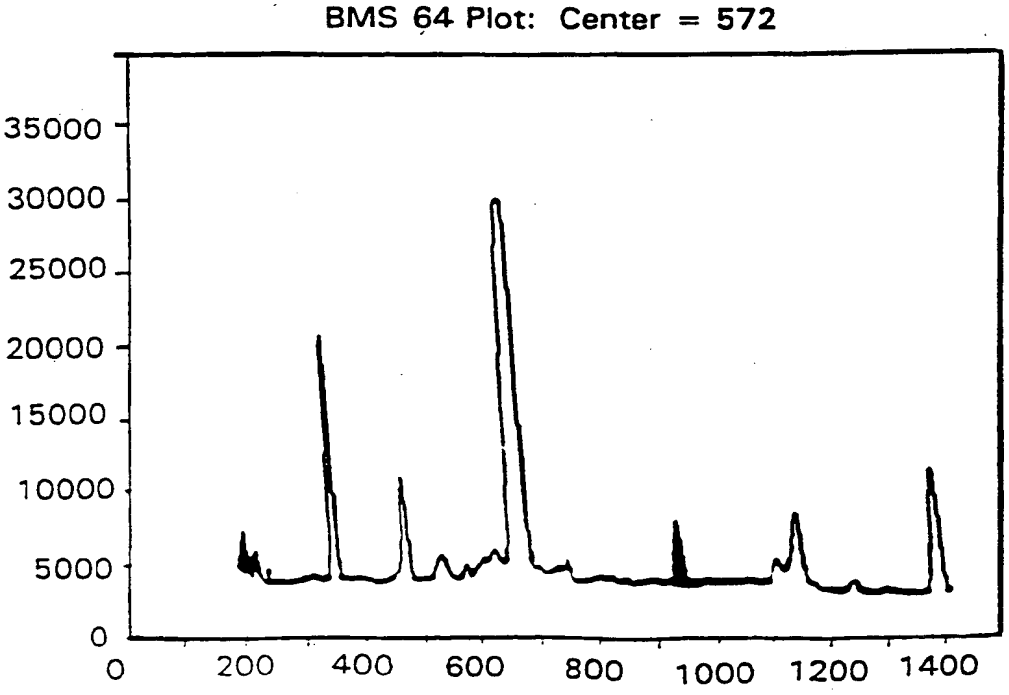

BMS 64 Plot: Center = 572
35000
30000
25000
20000
15000
10000
5000
0
0
200
400
600
800
1000
1200
1400

FIG.9(d)

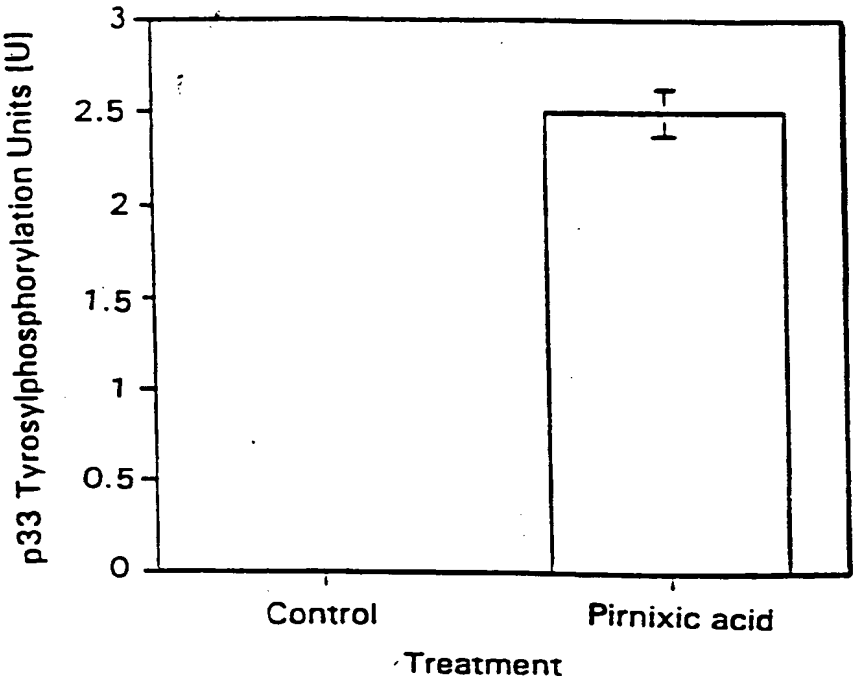

p33 Tyrosylphosphorylation Units (U)
3
2.5
2
1.5
1
0.5
0
Control
Pirnixic acid
Treatment

FIG.10

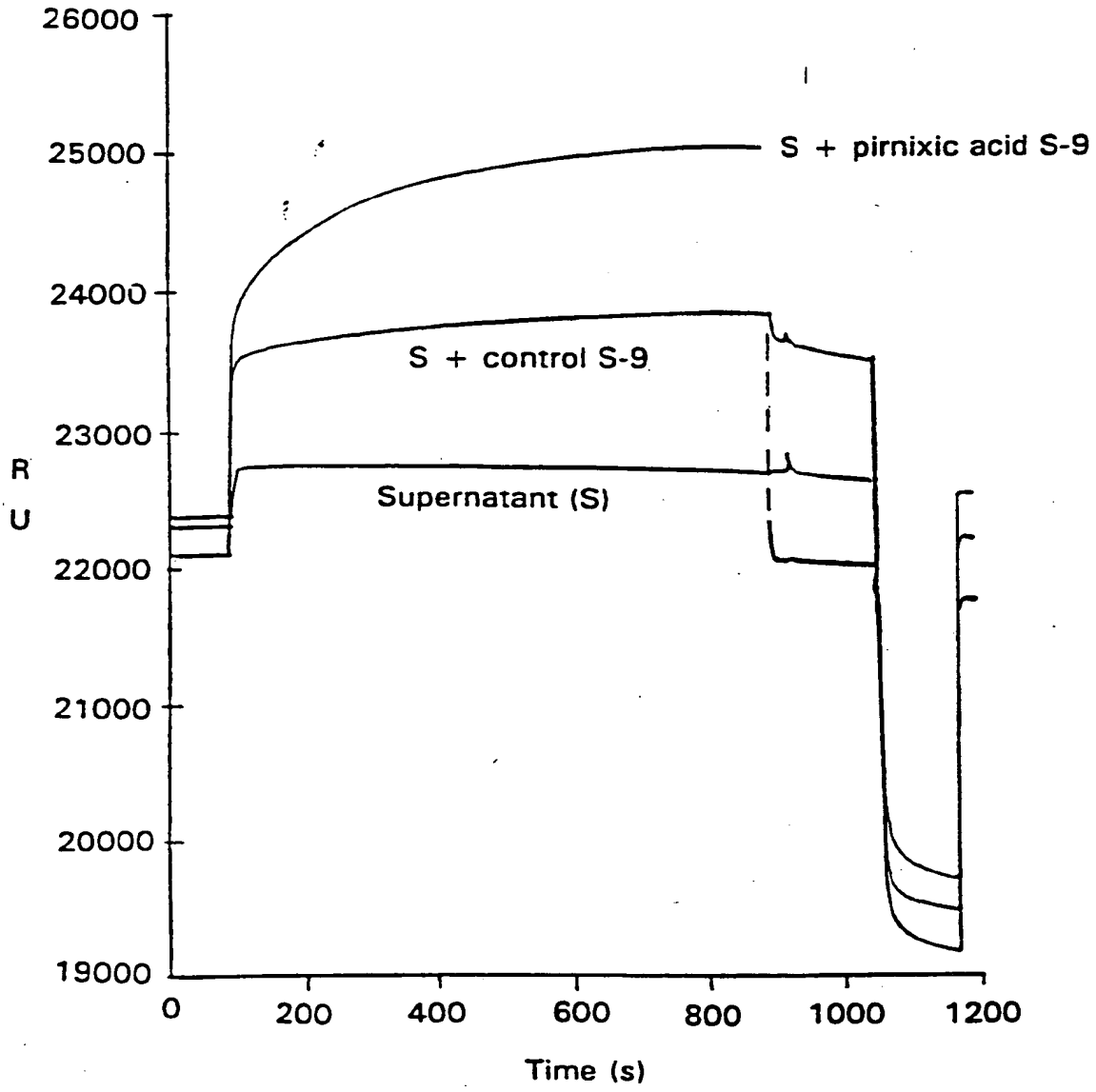

26000
25000
24000
23000
22000
21000
20000
19000
R
U
S + pirnixic acid S-9
S + control S-9
Supernatant (S)
0
200
400
600
800
1000
1200
Time (s)

FIG. 11

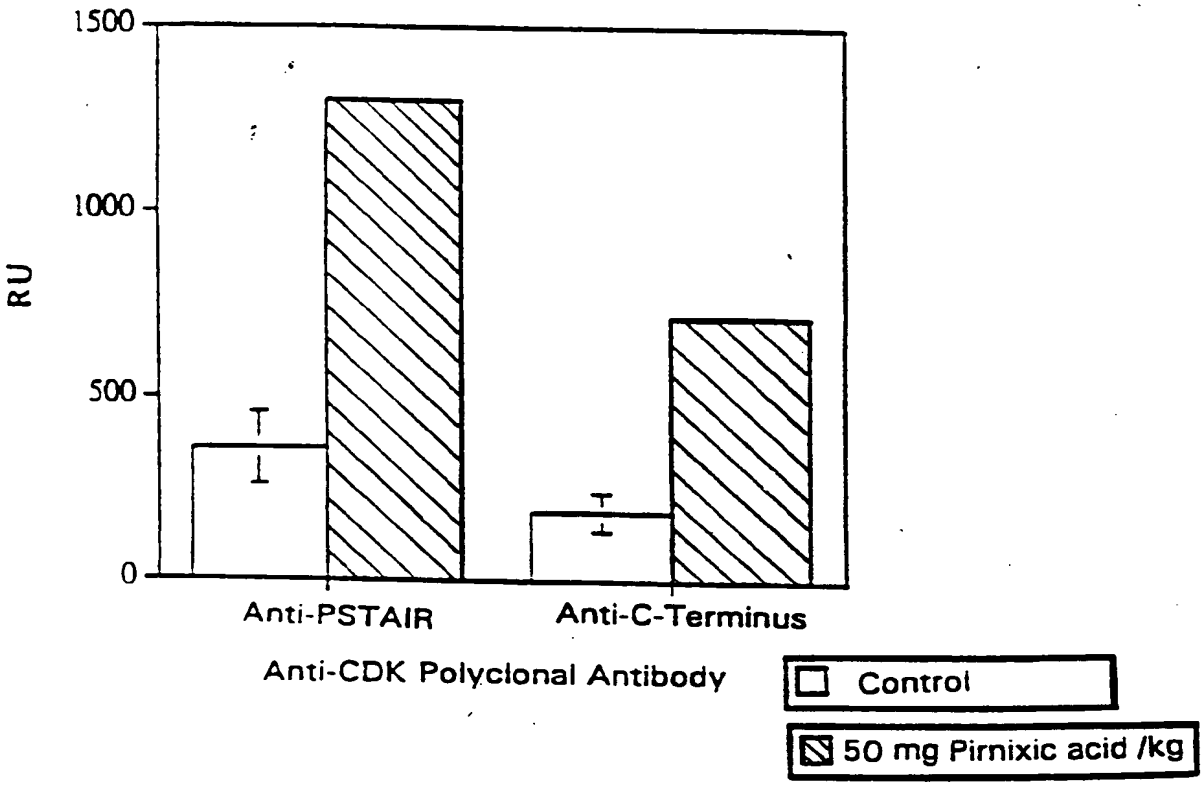

1500
1000
500
0
RU
Anti-PSTAIR
Anti-C-Terminus
Anti-CDK Polyclonal Antibody
Control
50 mg Pirnixic acid /kg

FIG. 12

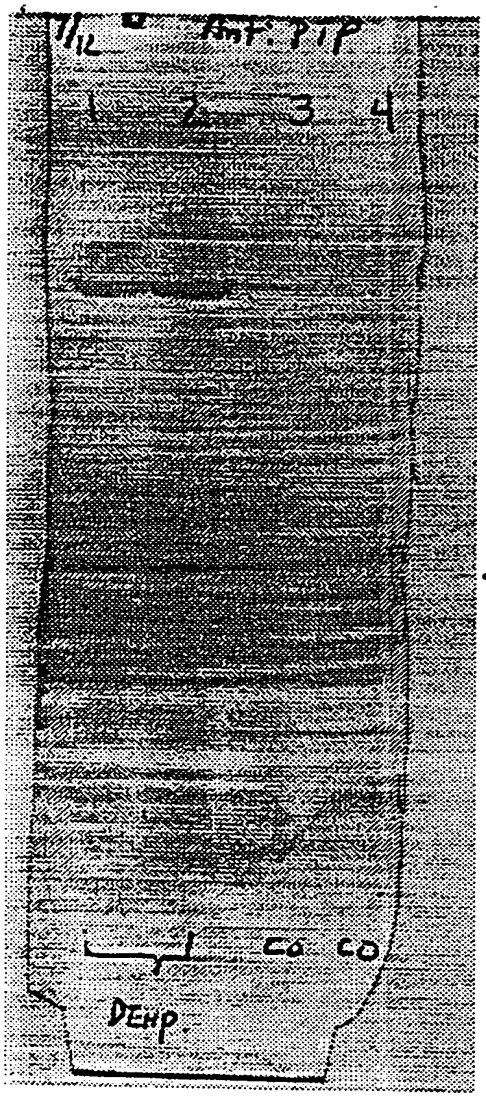


FIG. 13

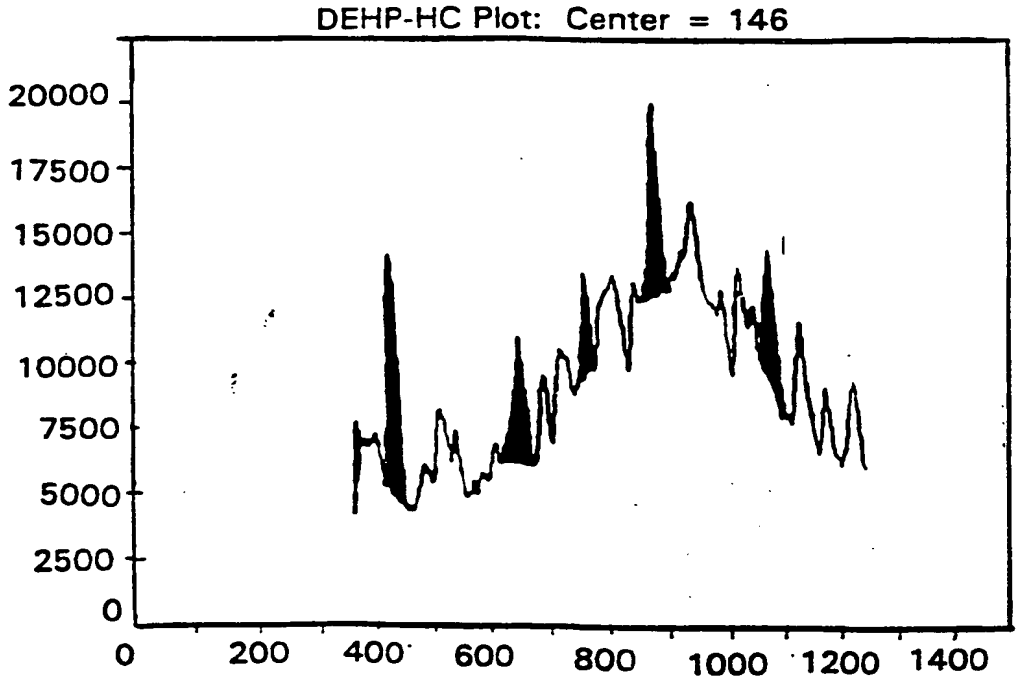


FIG. 14(a)

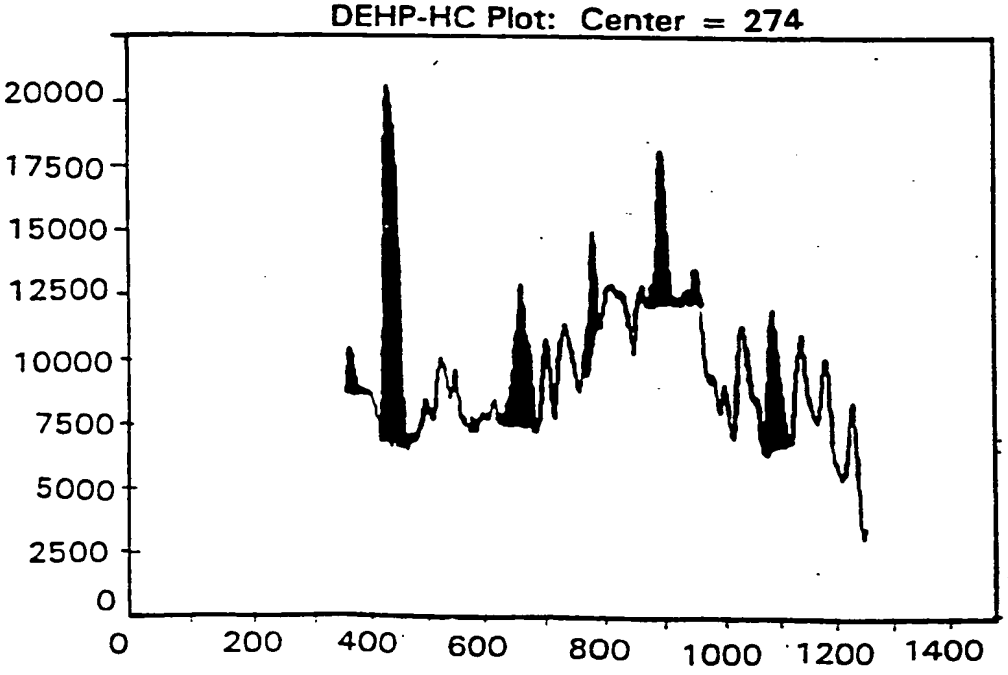


FIG. 14(b)

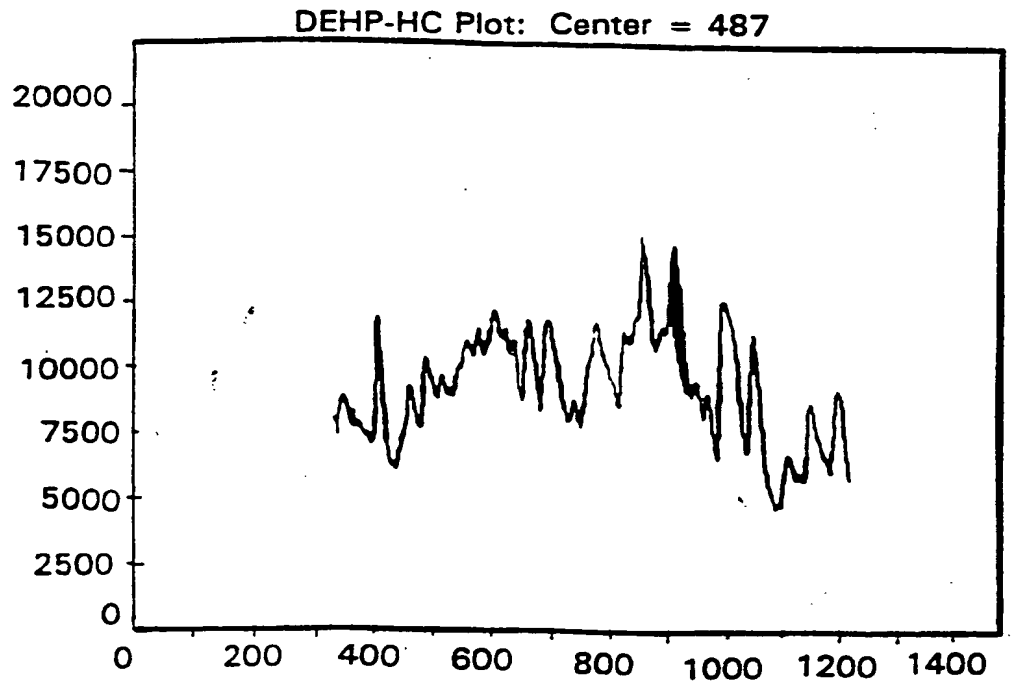


FIG. 14 (c)

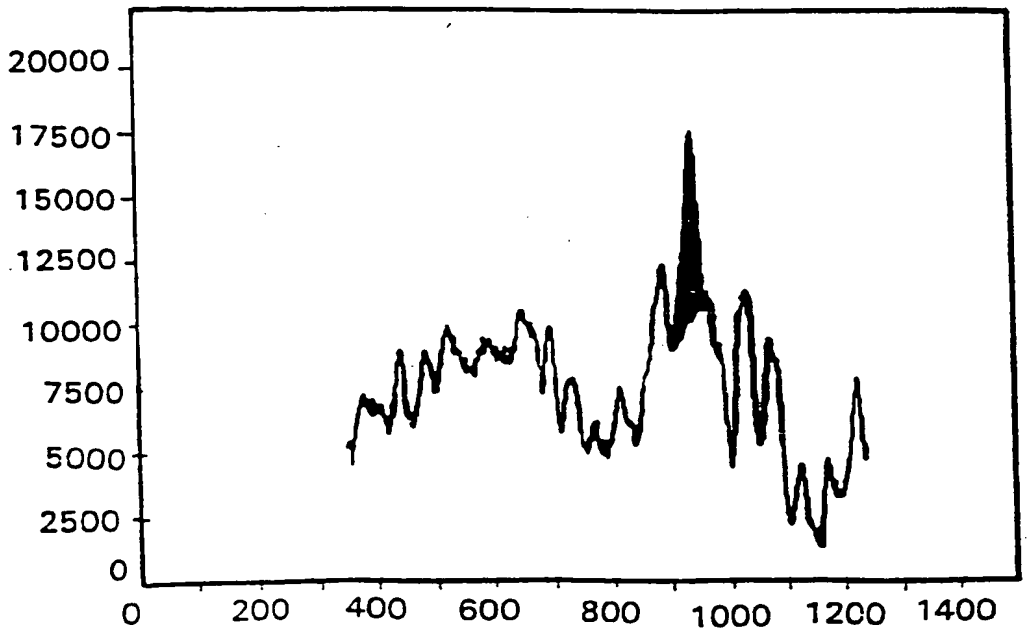


FIG. 14 (d)

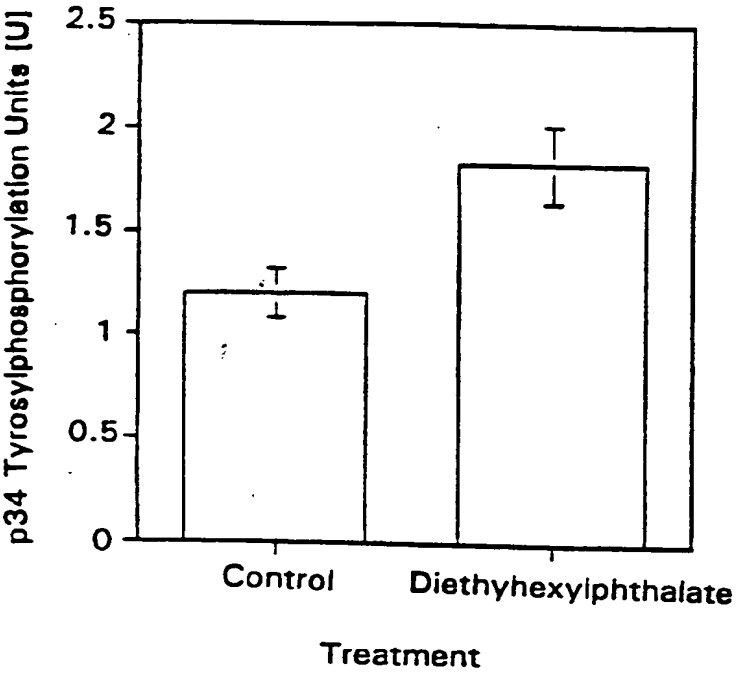

p34 Tyrosylphosphorylation Units [U]
2.5
2
1.5
1
0.5
0
Control
Diethyhexylphthalate
Treatment

FIG.15

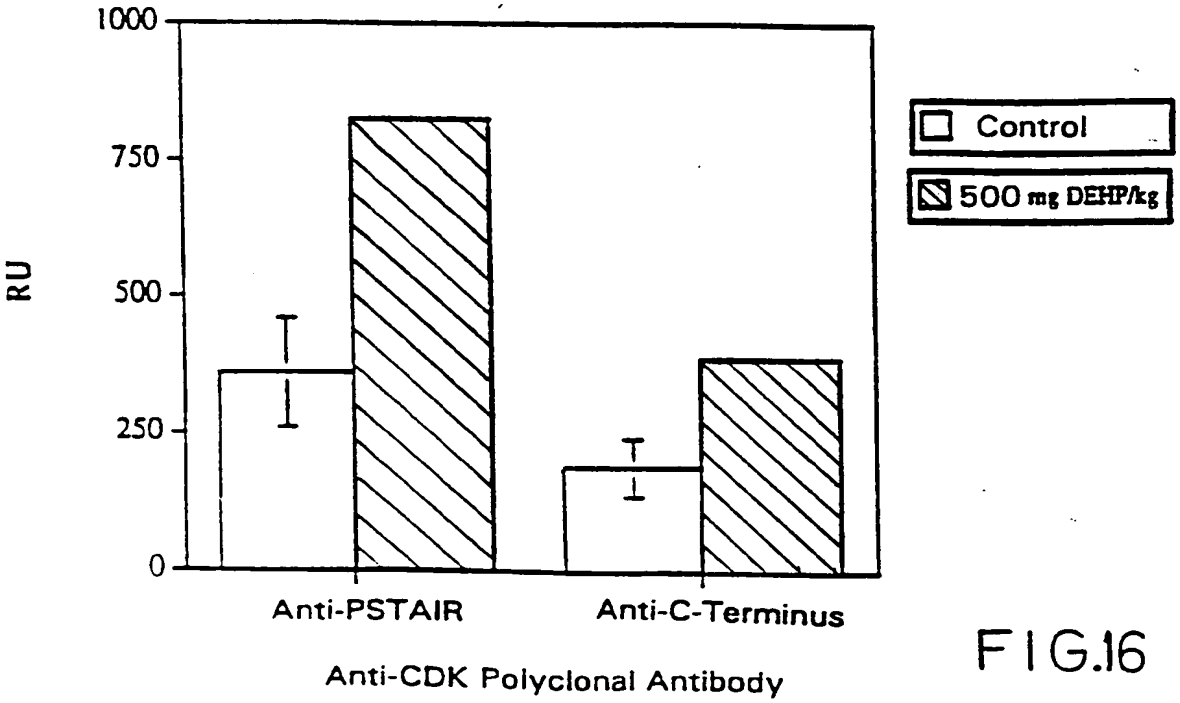

RU
1000
750
500
250
0
Anti-PSTAIR
Anti-C-Terminus
Anti-CDK Polyclonal Antibody
Control
500 mg DEHP/kg

FIG.16

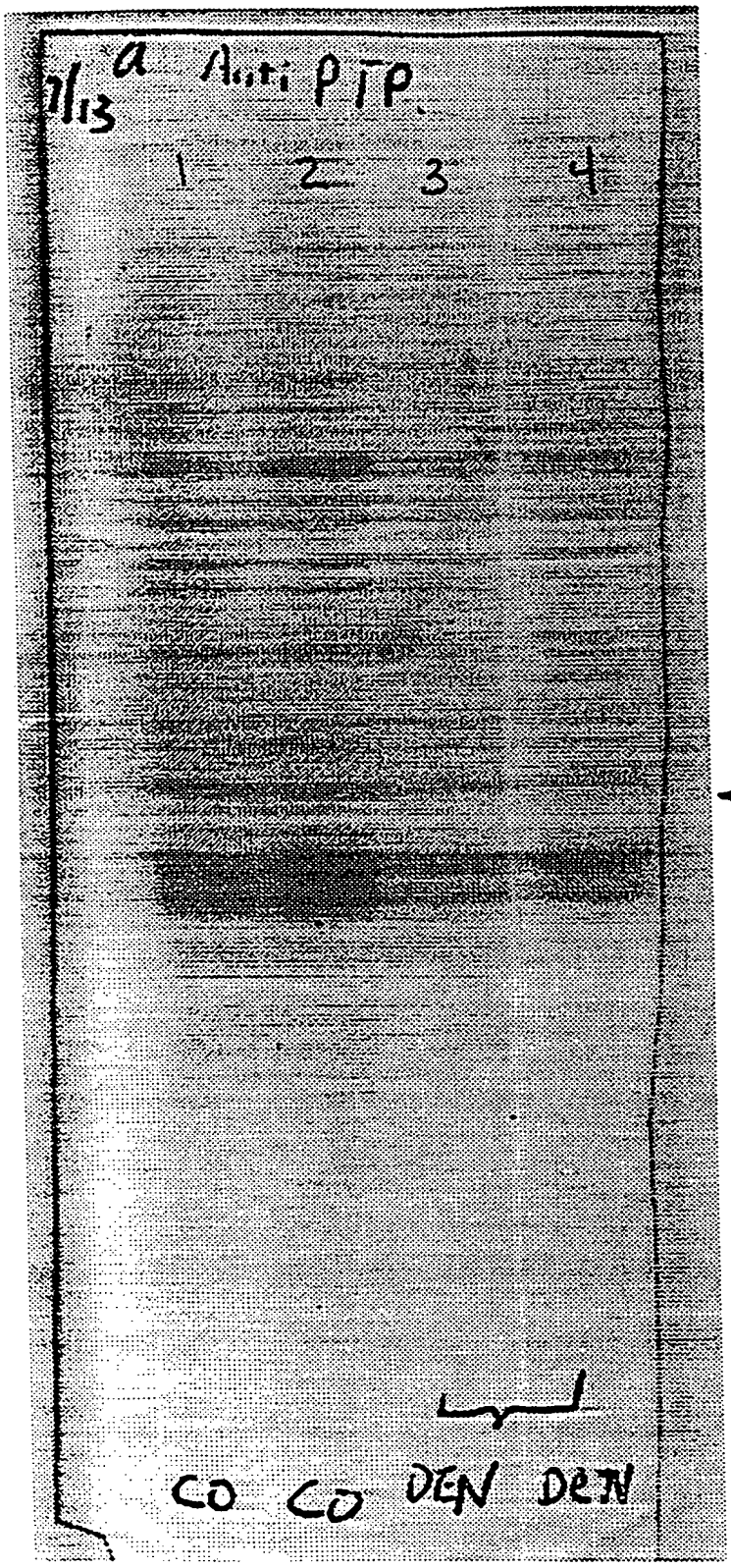


FIG. 17

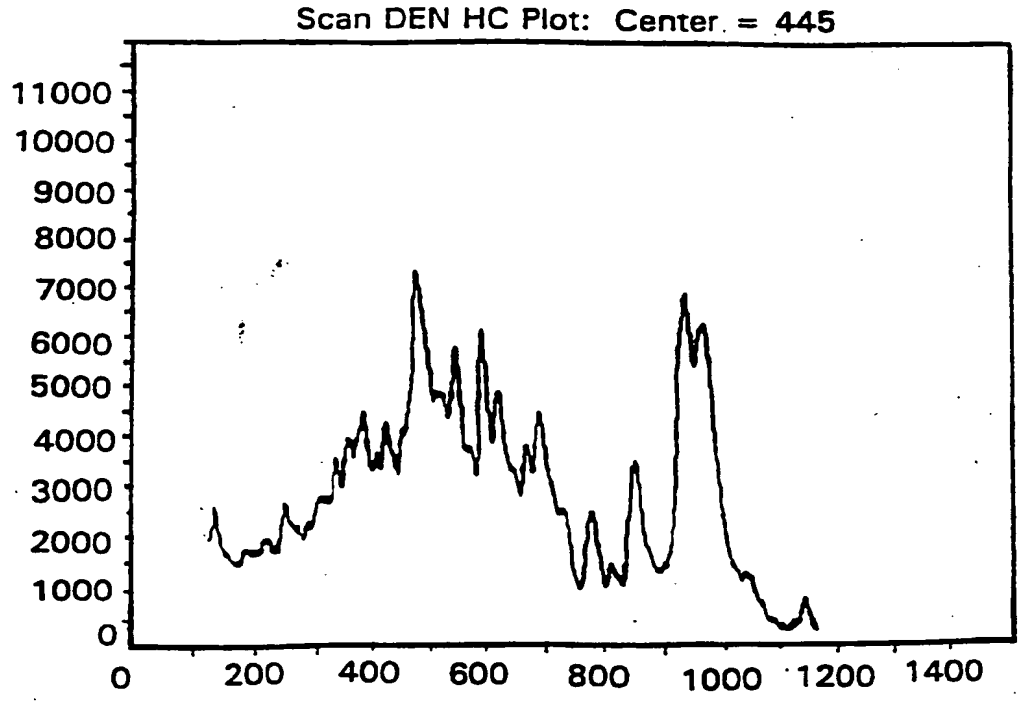


FIG. 18(a)

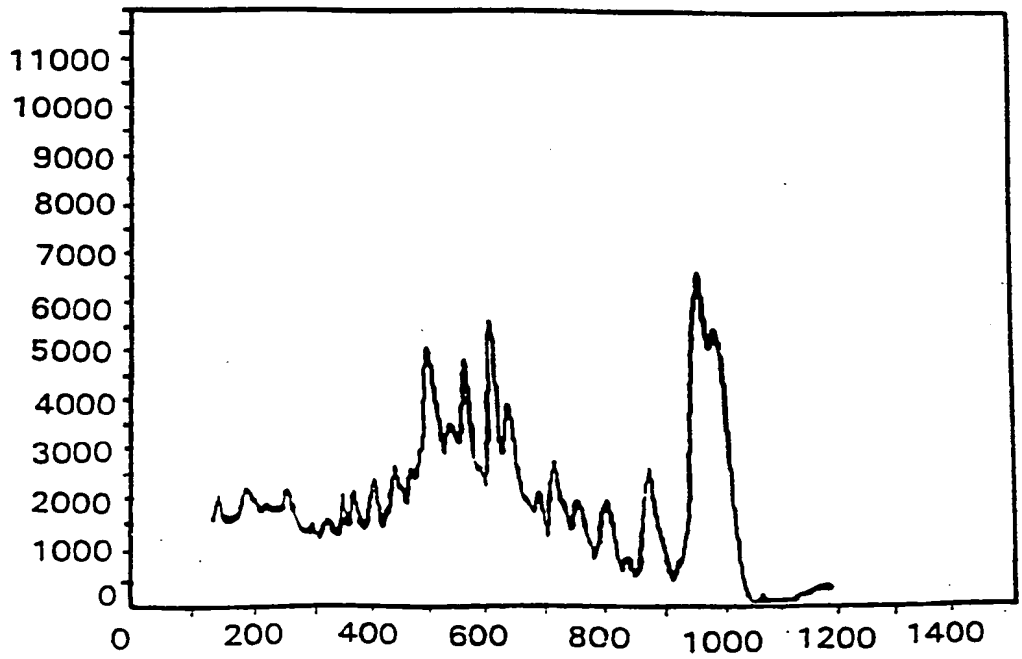


FIG. 18(b)

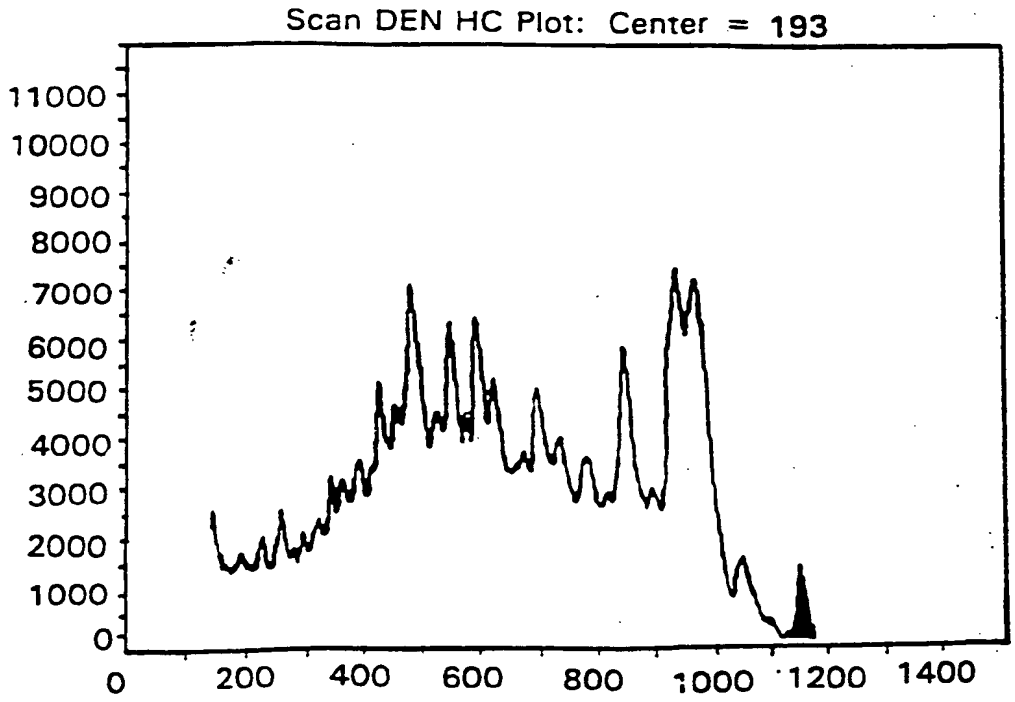


FIG. 18(c)

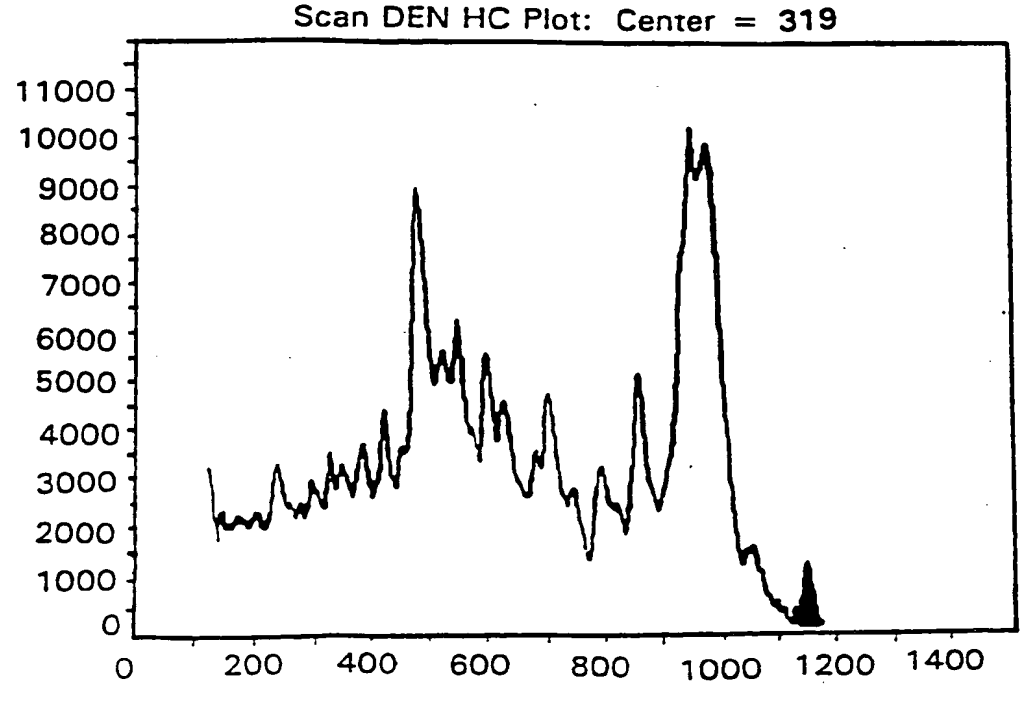


FIG. 18(d)

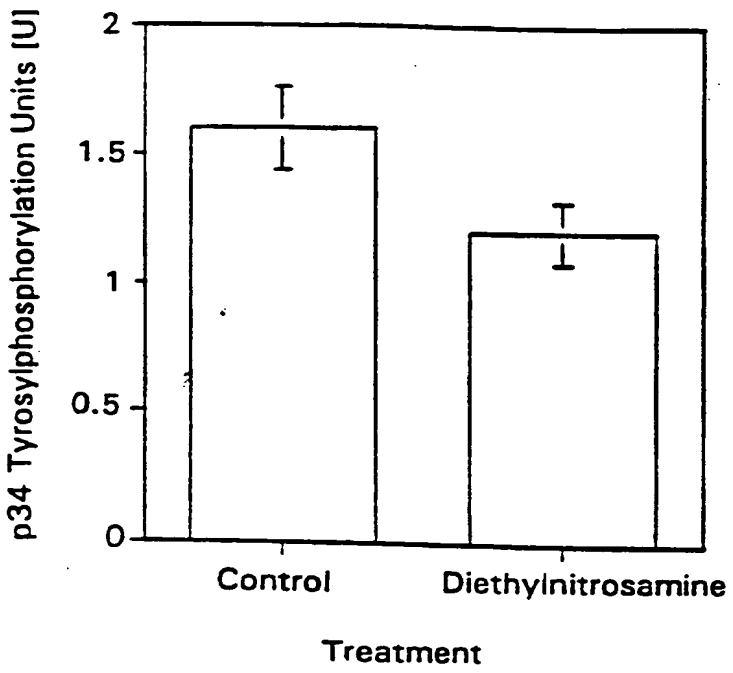

p34 Tyrosylphosphorylation Units [U]
2
1.5
1
0.5
0
Control
Diethylnitrosamine
Treatment

FIG.19

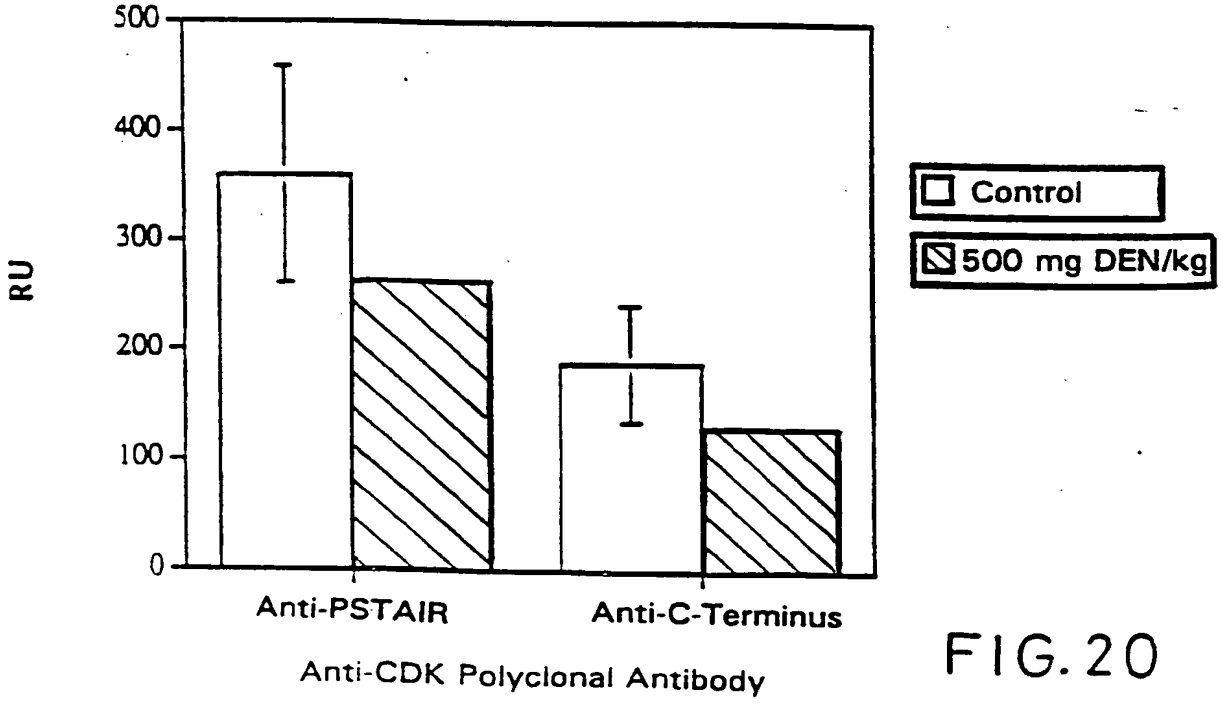
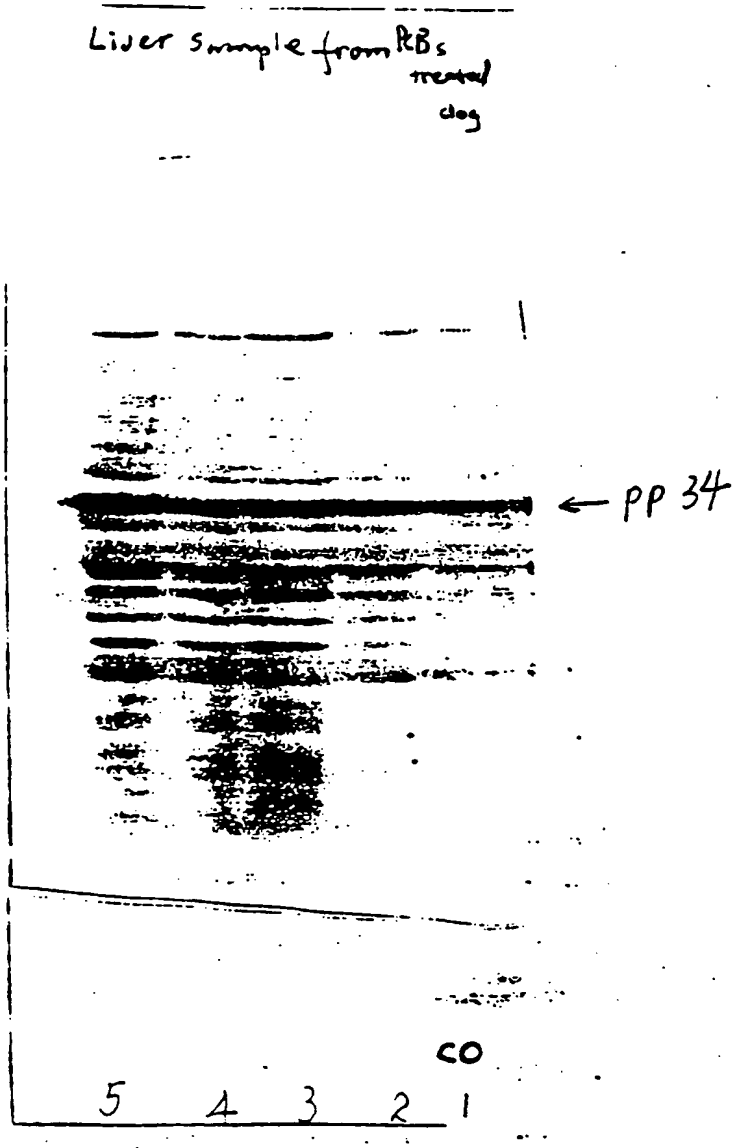

RU
500
400
300
200
100
0
Anti-PSTAIR
Anti-C-Terminus
Anti-CDK Polyclonal Antibody
Control
500 mg DEN/kg

FIG. 20

FIG. 21



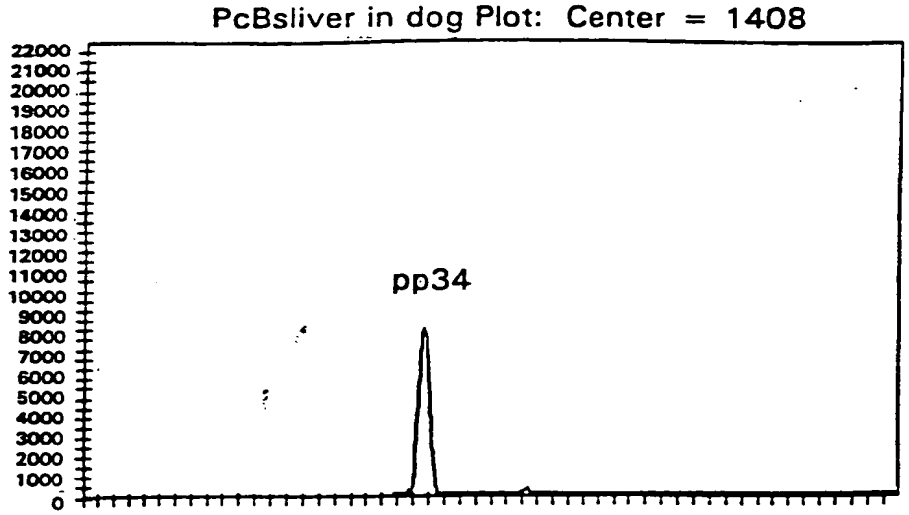


FIG. 22(a)

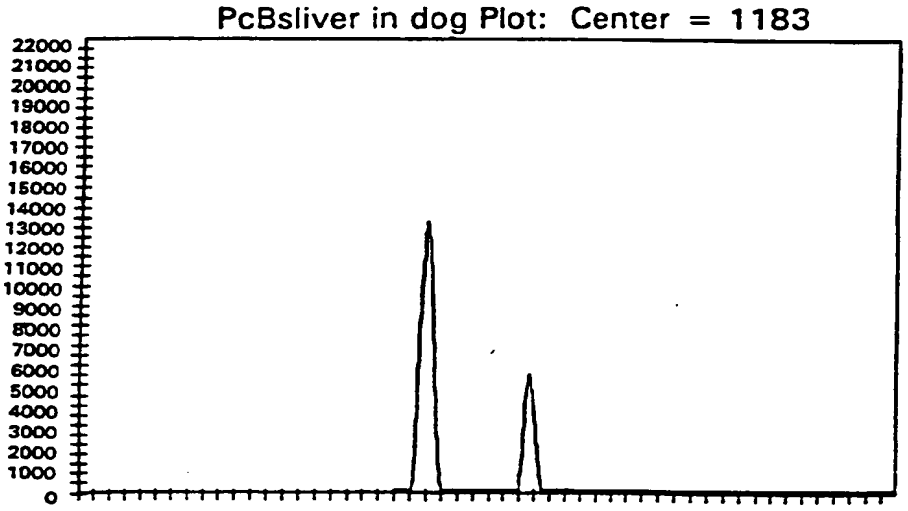


FIG. 22(b)

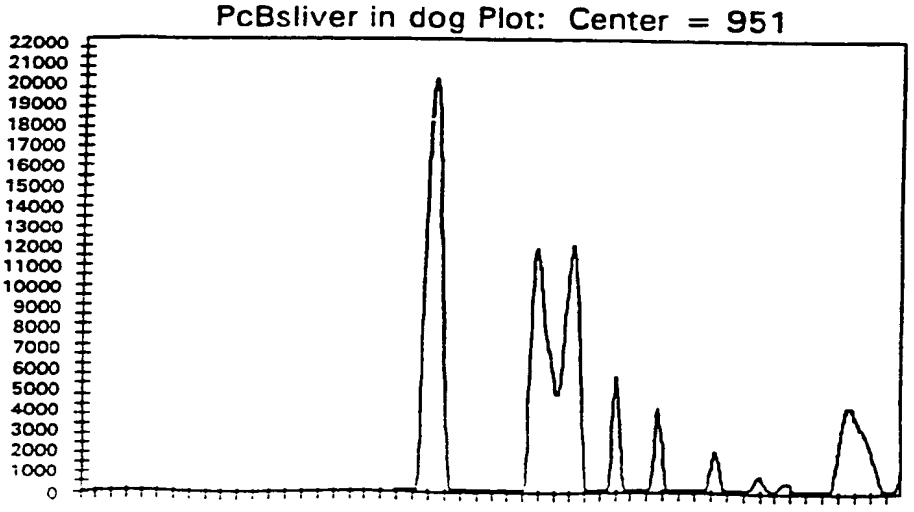


FIG. 22(c)

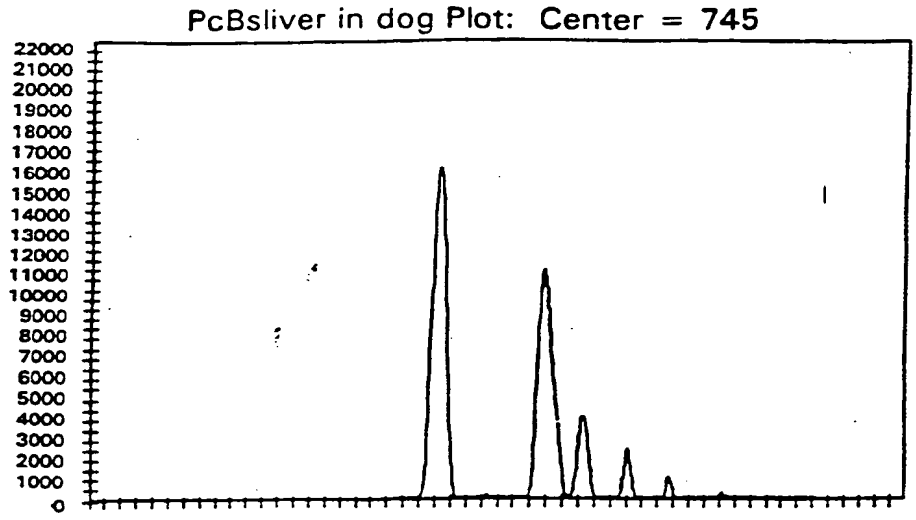


FIG. 22(d)

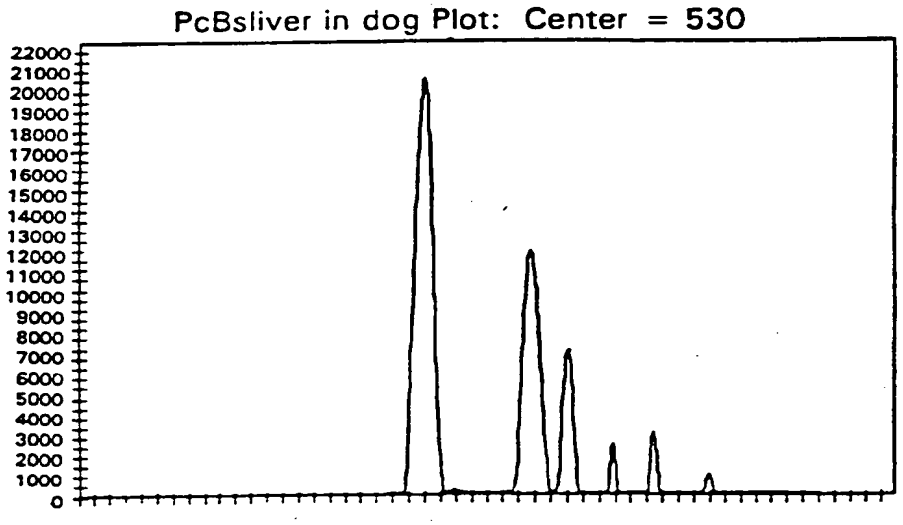


FIG. 22(e)

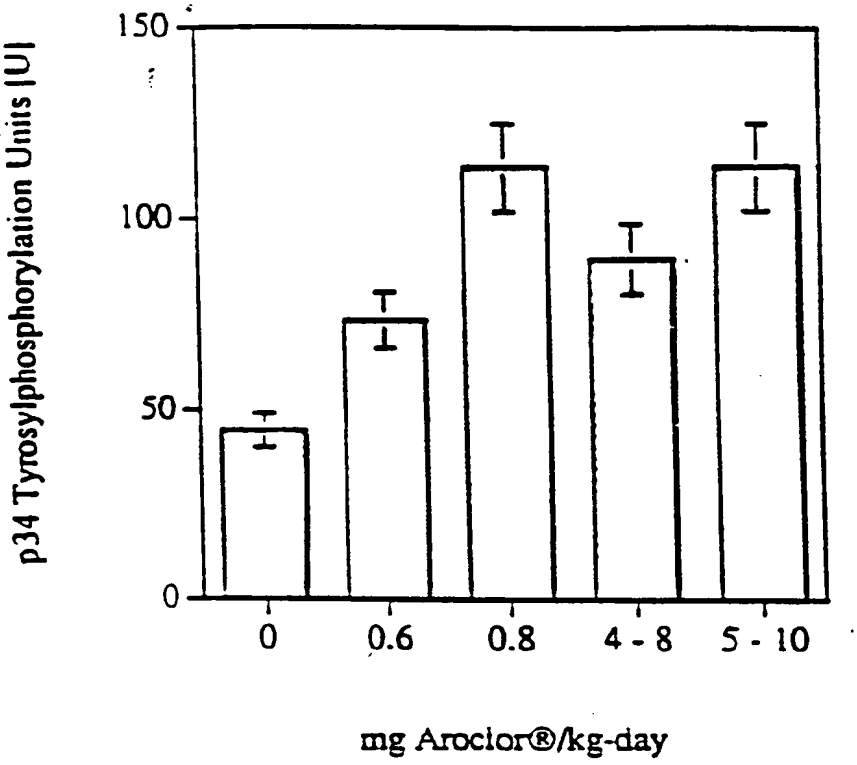

p34 Tyrosylphosphorylation Units (U)
150
100
50
0
0
0.6
0.8
4 - 8
5 - 10
mg Aroclor®/kg-day

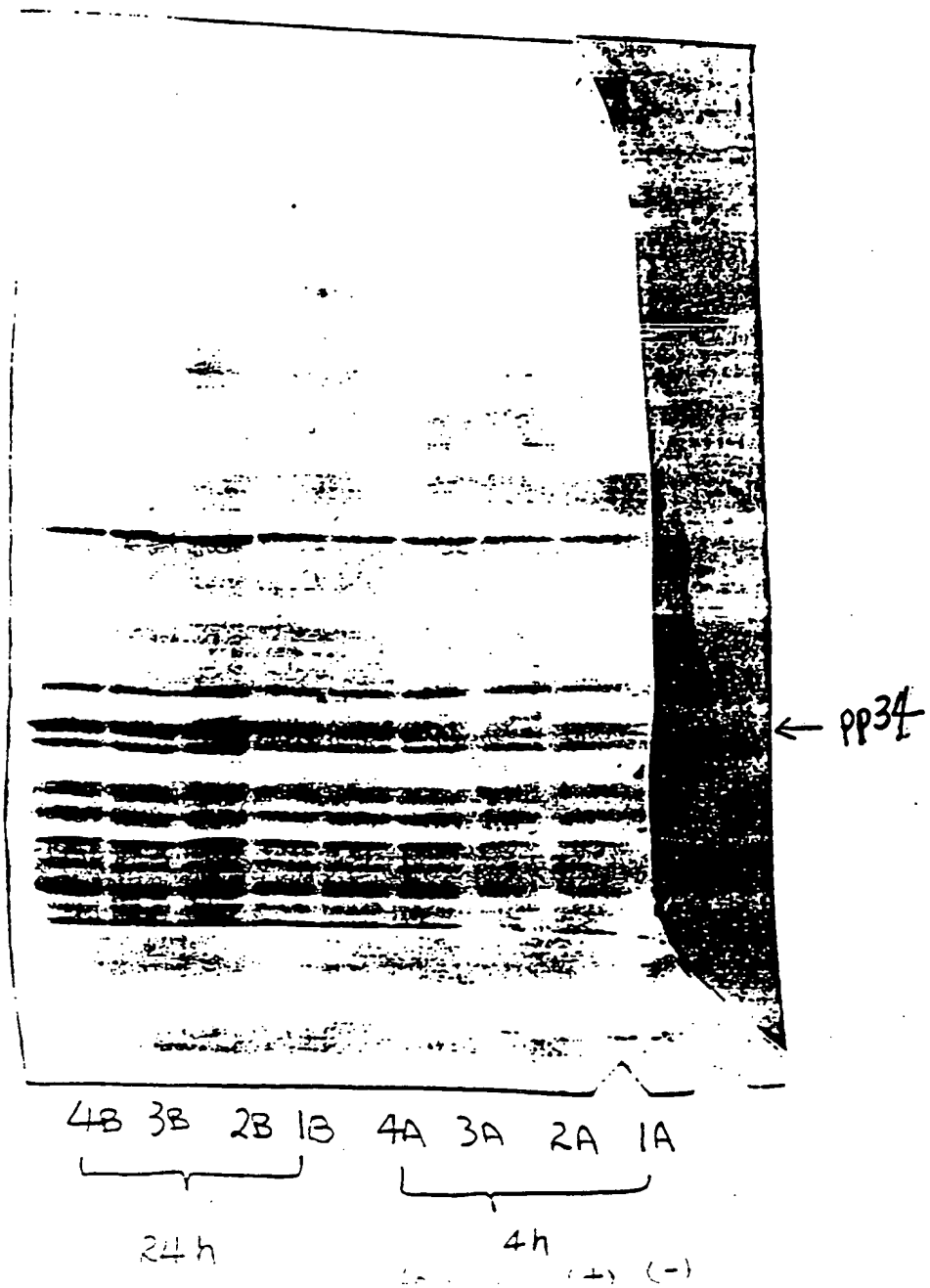
FIG. 23

24/44

FIG. 24

3T3 cell line

with Anti-phosphorylation tyrosine Antibody



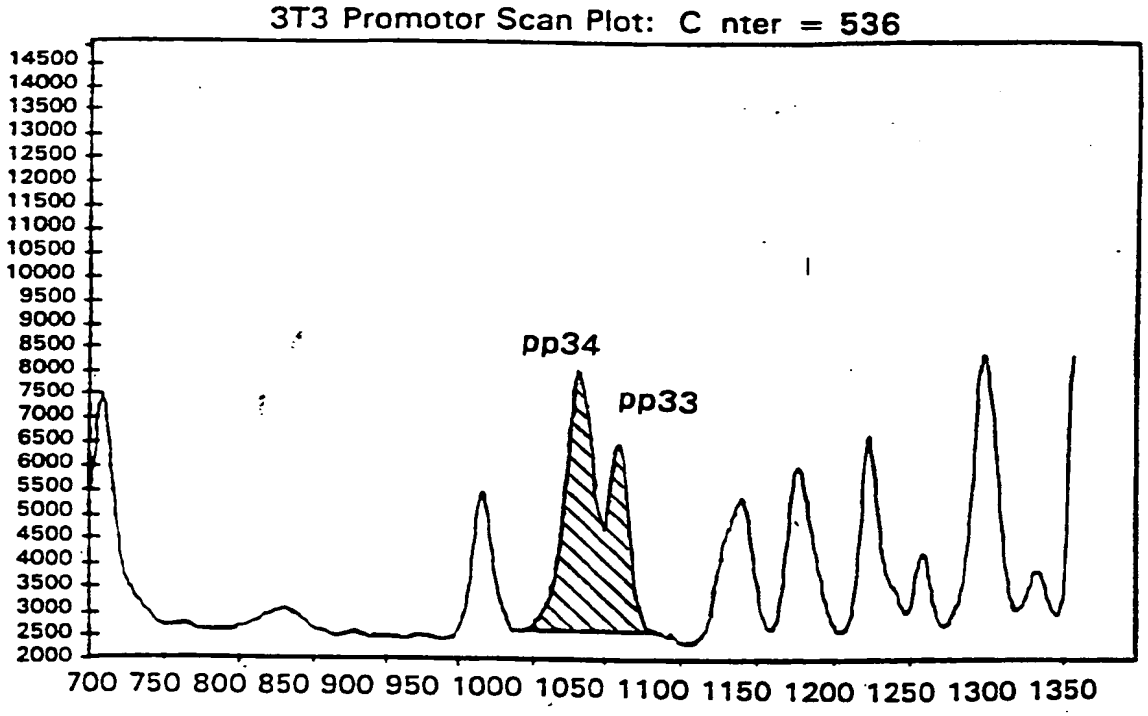


FIG. 25(a)

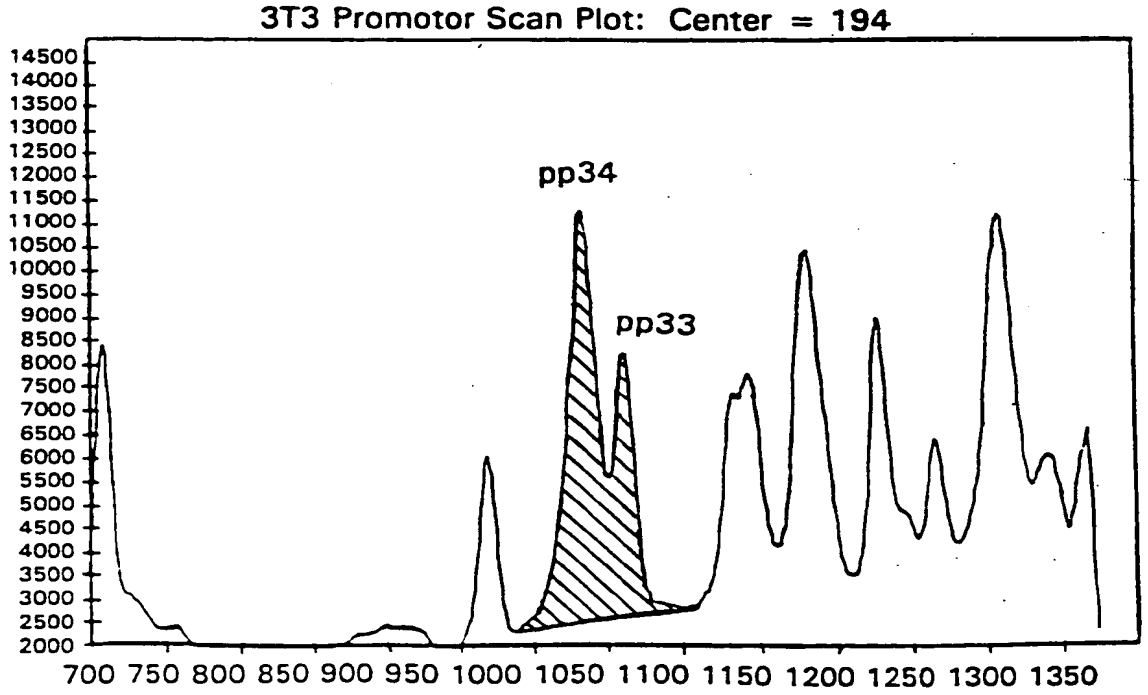


FIG. 25(b)

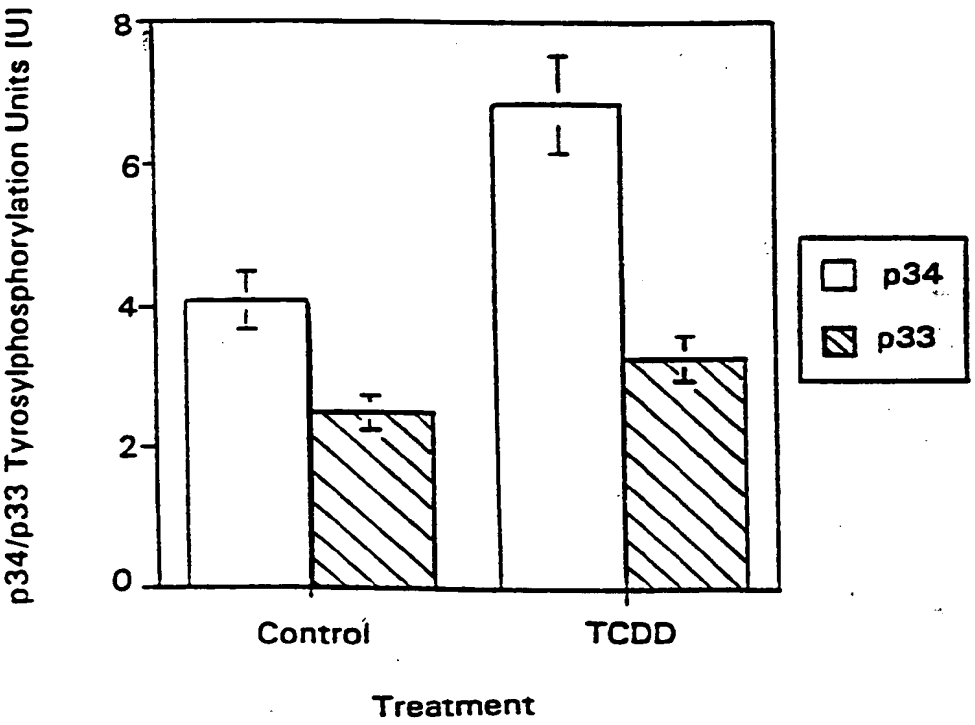
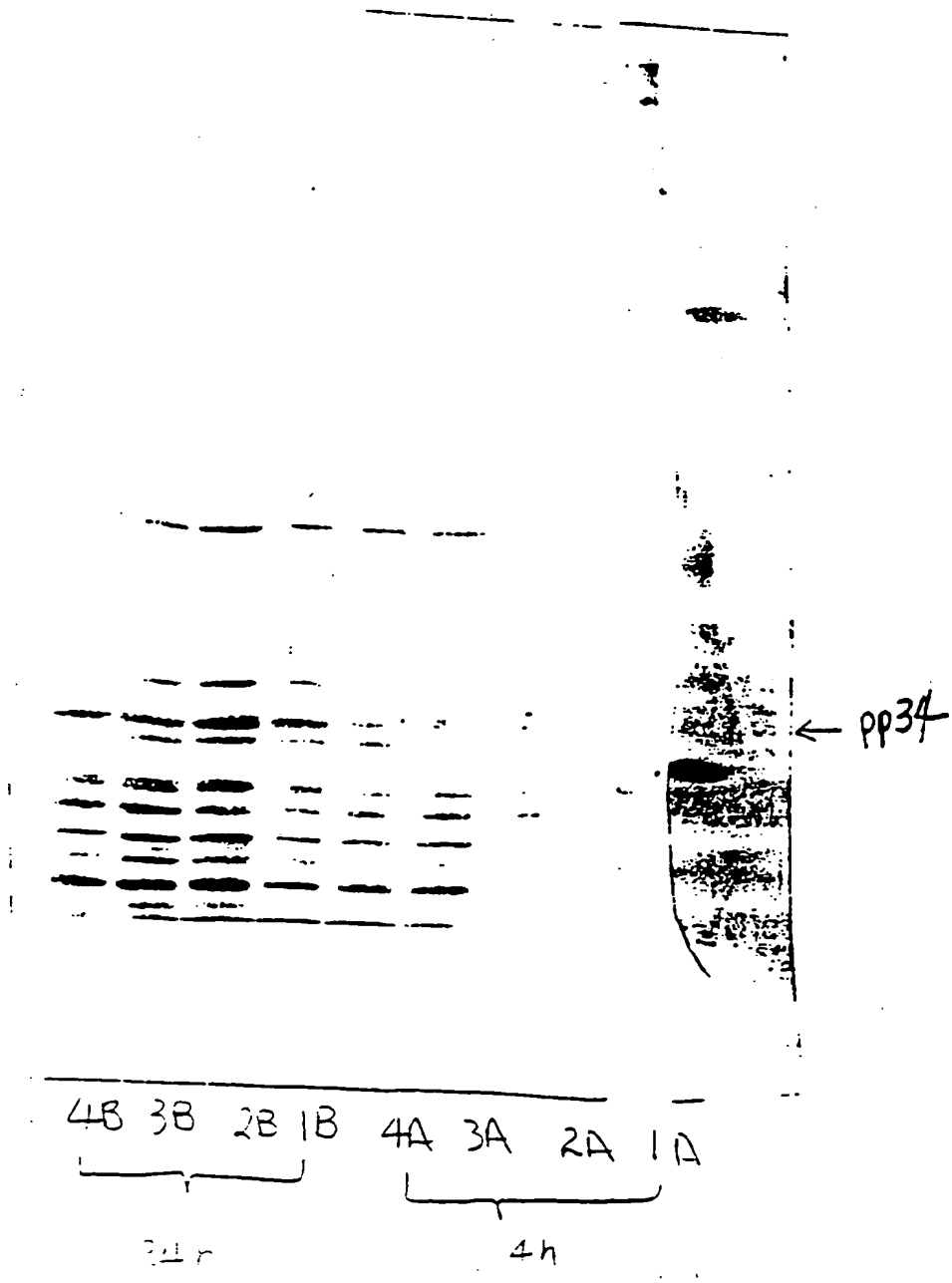

p34/p33 Tyrosylphosphorylation Units [U]
8
6
4
2
0
Control
TCDD
Treatment
p34
p33

FIG. 26

FIG. 27



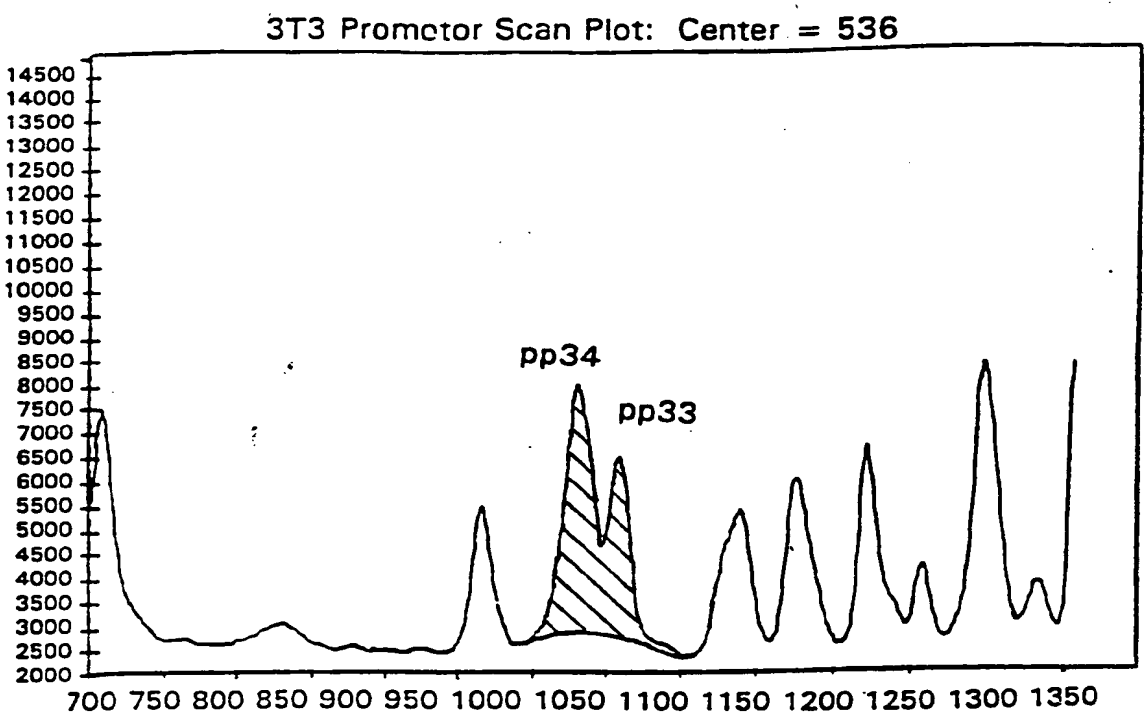


FIG. 28 (a)

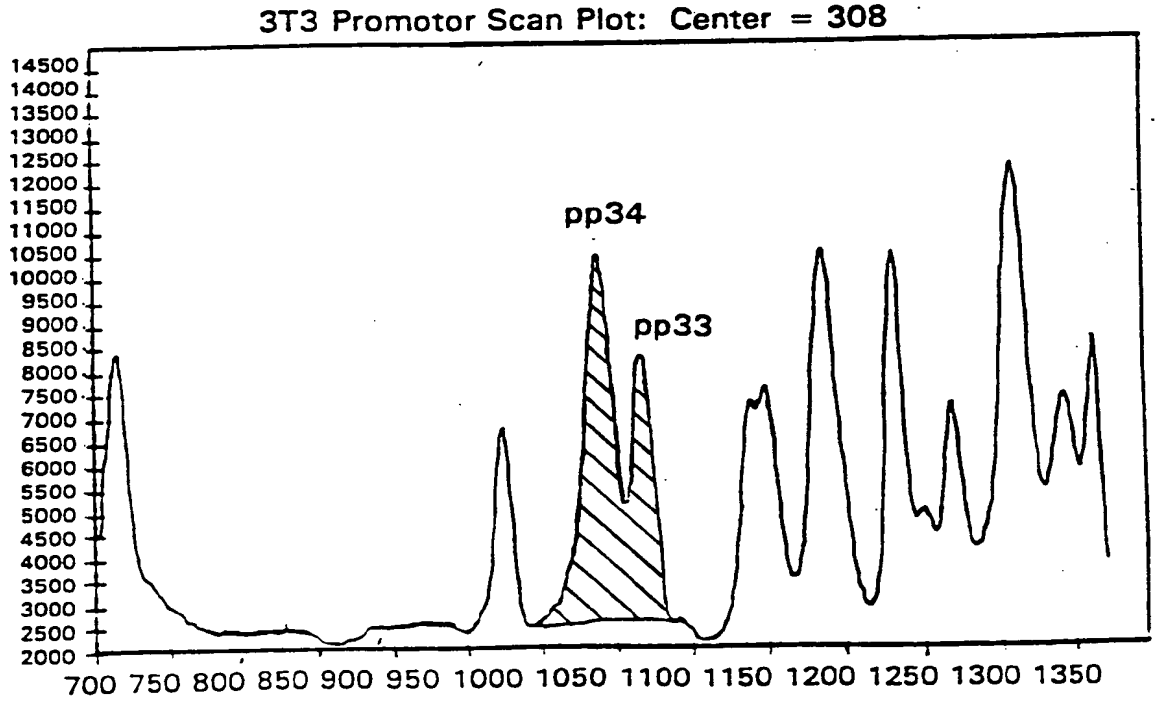


FIG. 28 (b)

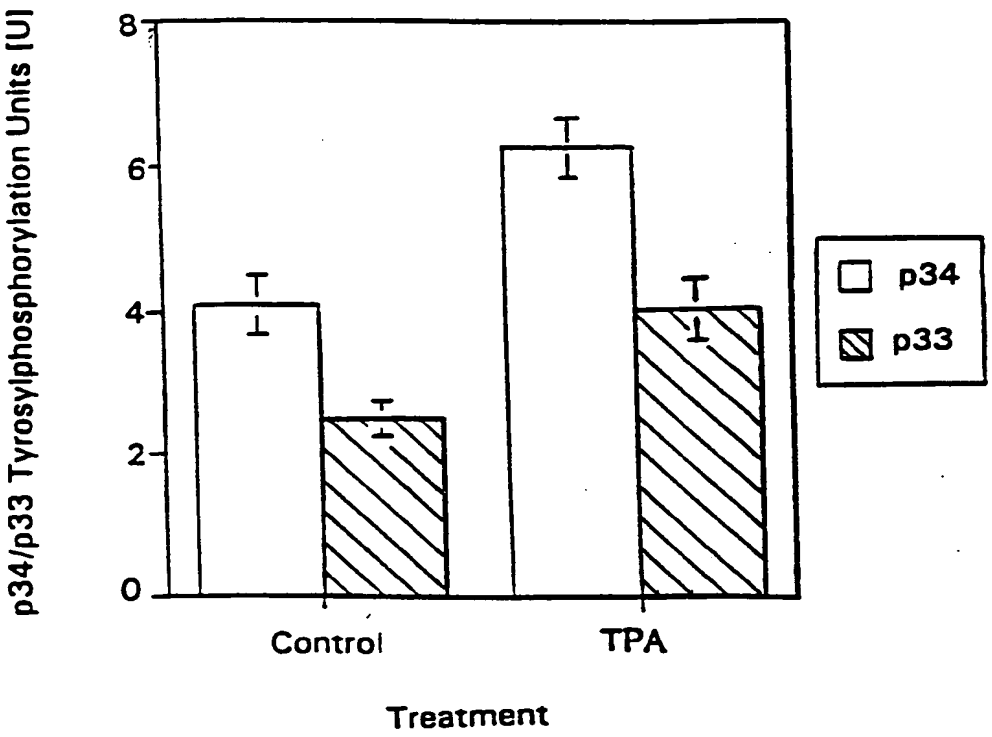

p34/p33 Tyrosylphosphorylation Units [U]
8
6
4
2
0
Control
TPA
Treatment
p34
p33

FIG. 29

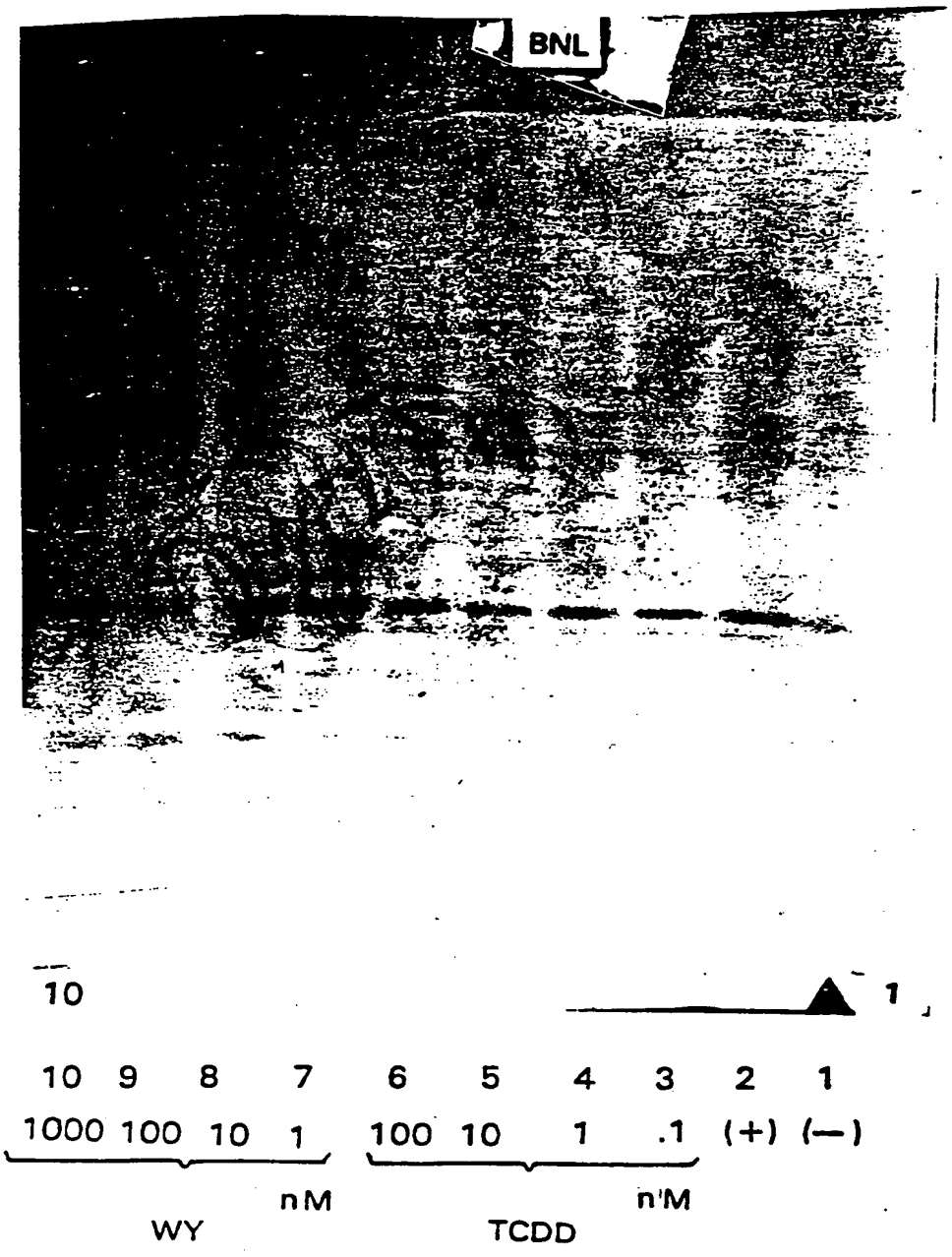


FIG. 30

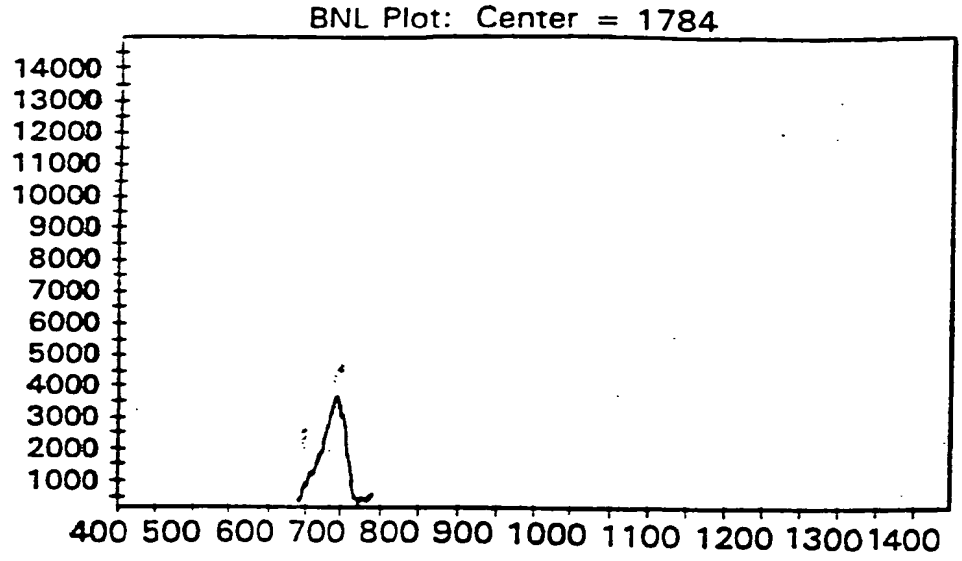


FIG. 31(a)

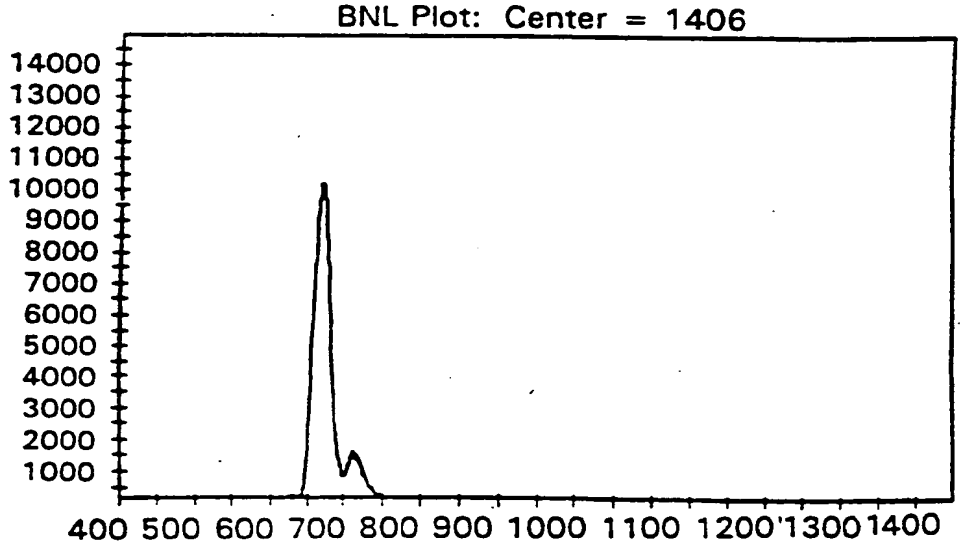


FIG. 31(c)

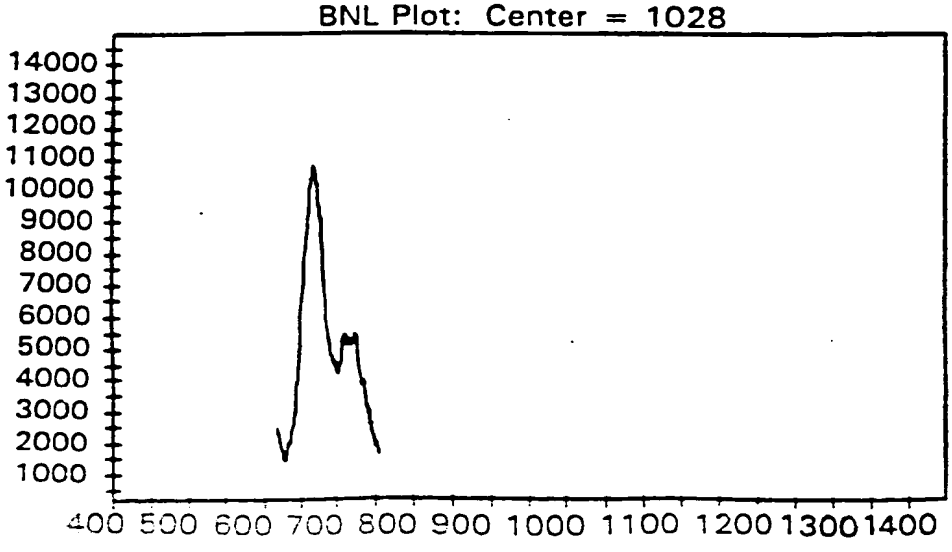


FIG. 31(e)

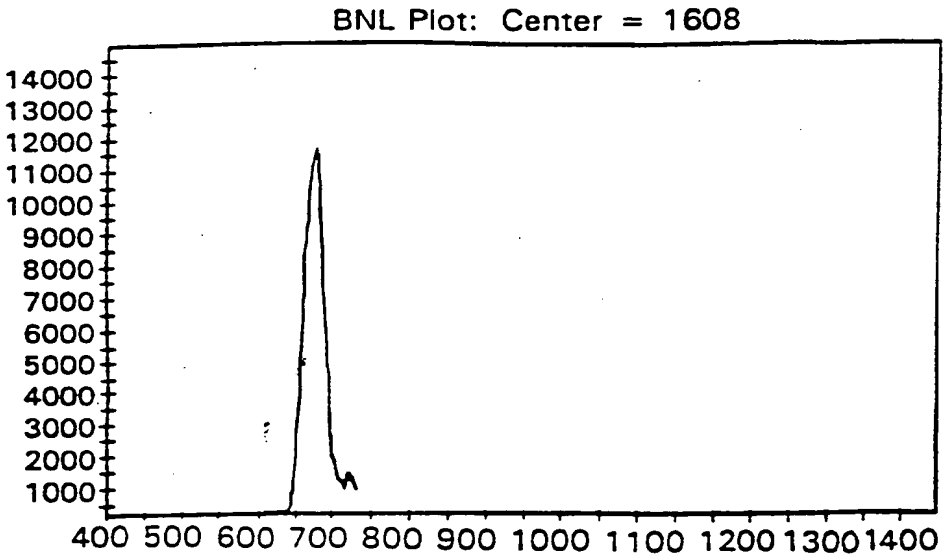


FIG. 31(b)

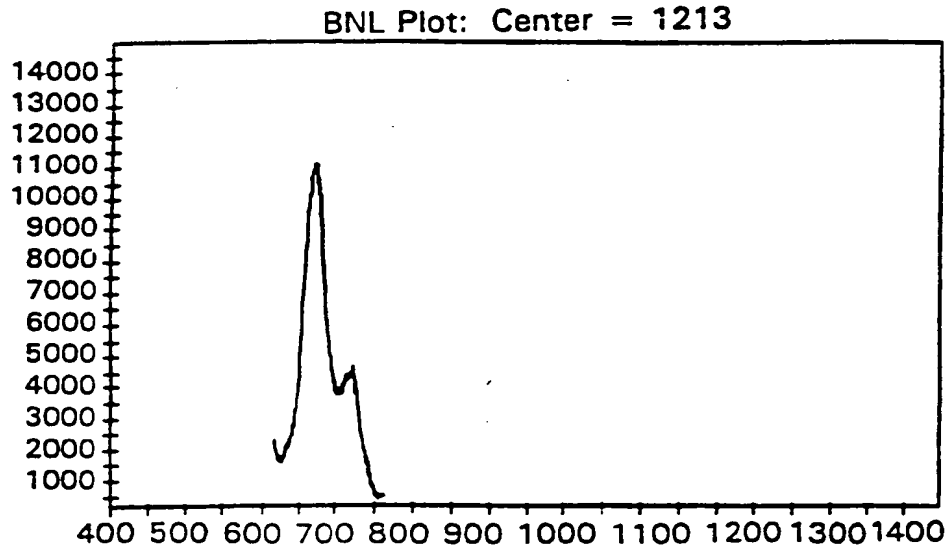


FIG. 31(d)

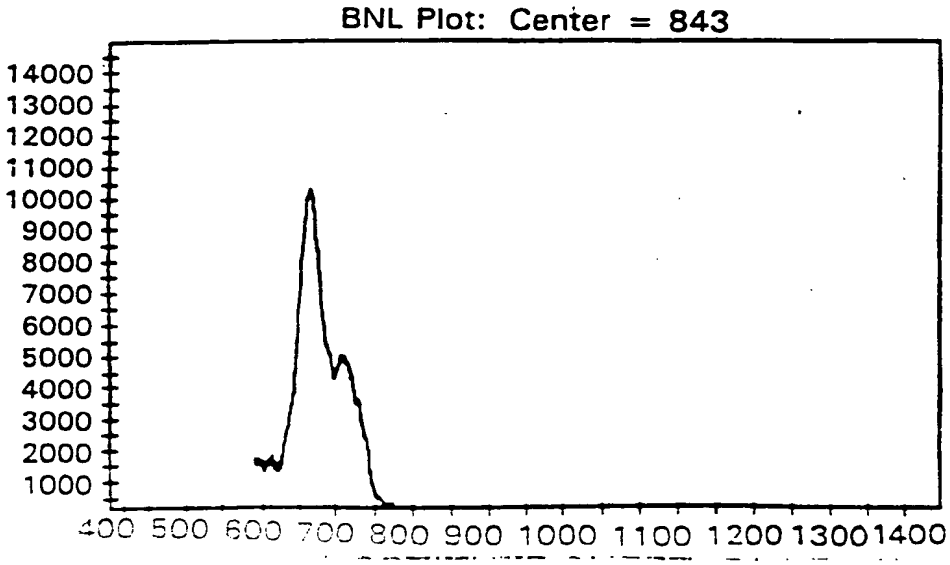


FIG. 31(f)

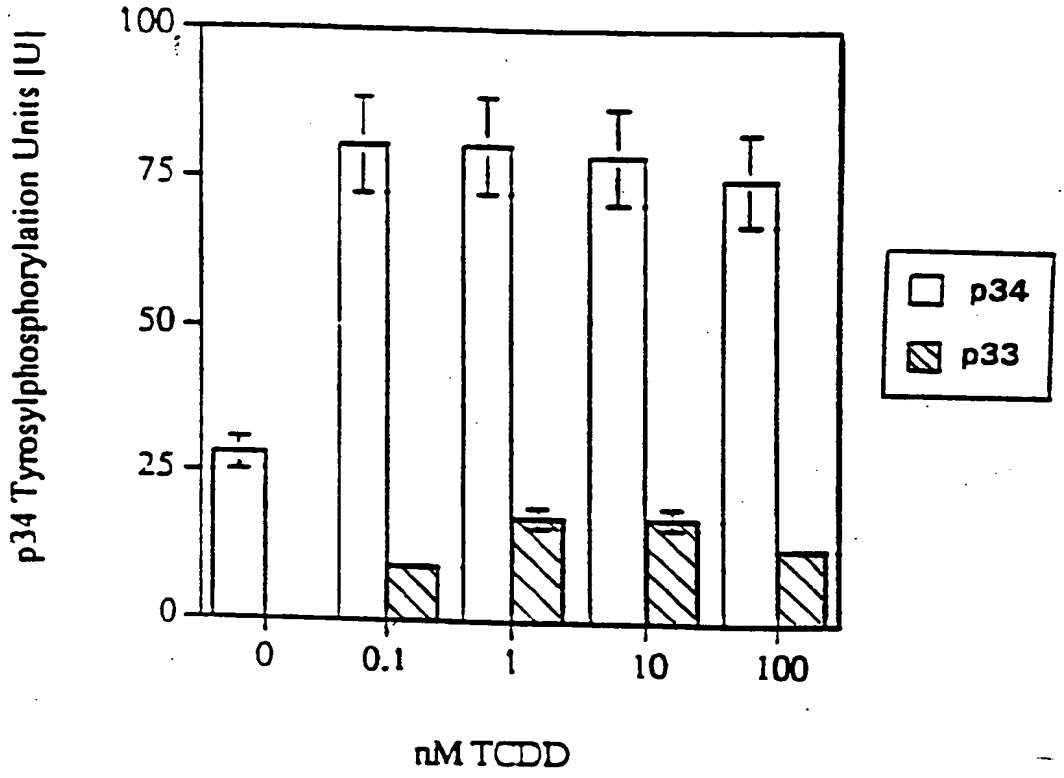

p34 Tyrosylphosphorylation Units [U]
100
75
50
25
0
0
0.1
1
10
100
nM TCDD
p34
p33

FIG. 32

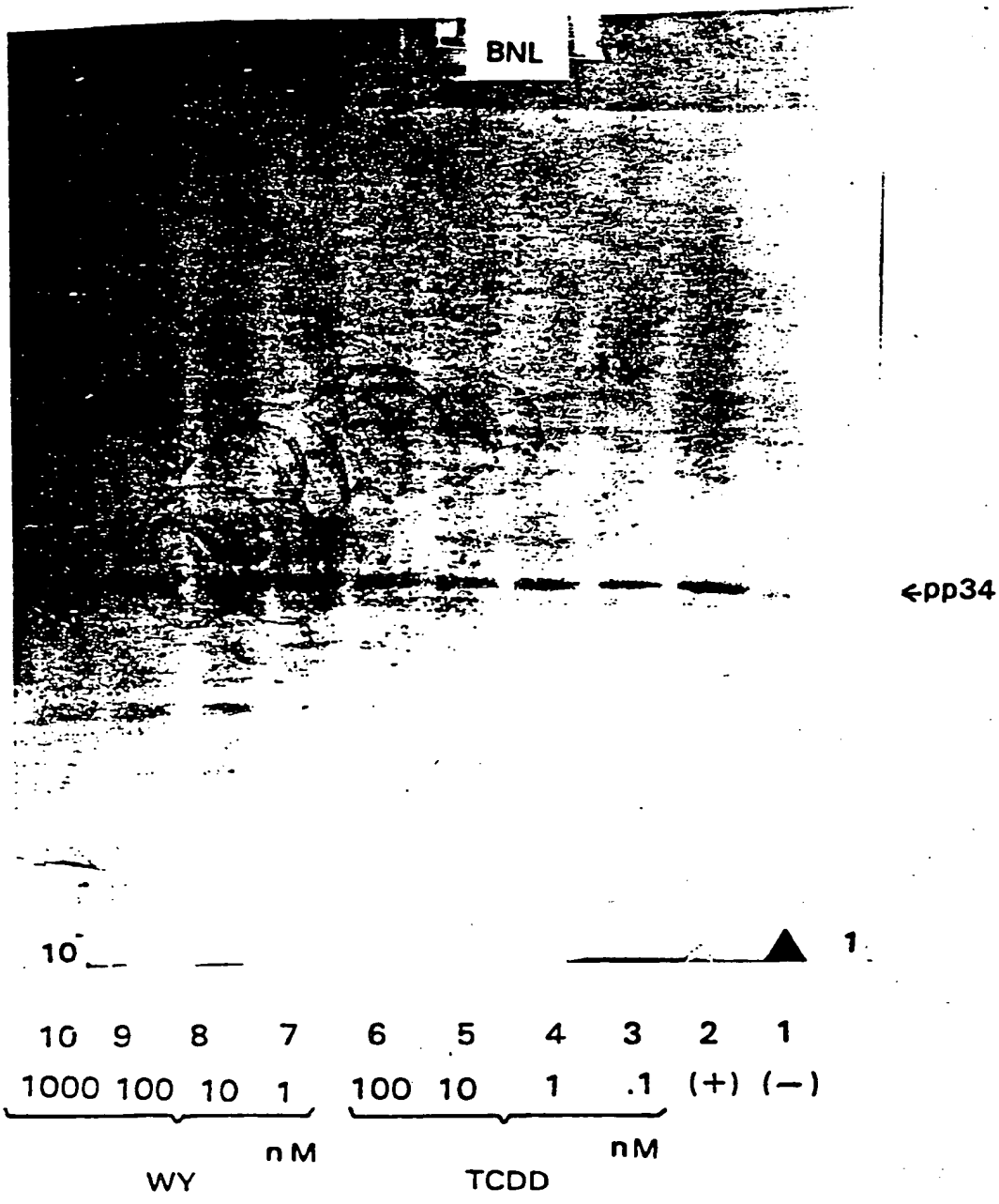


FIG. 33

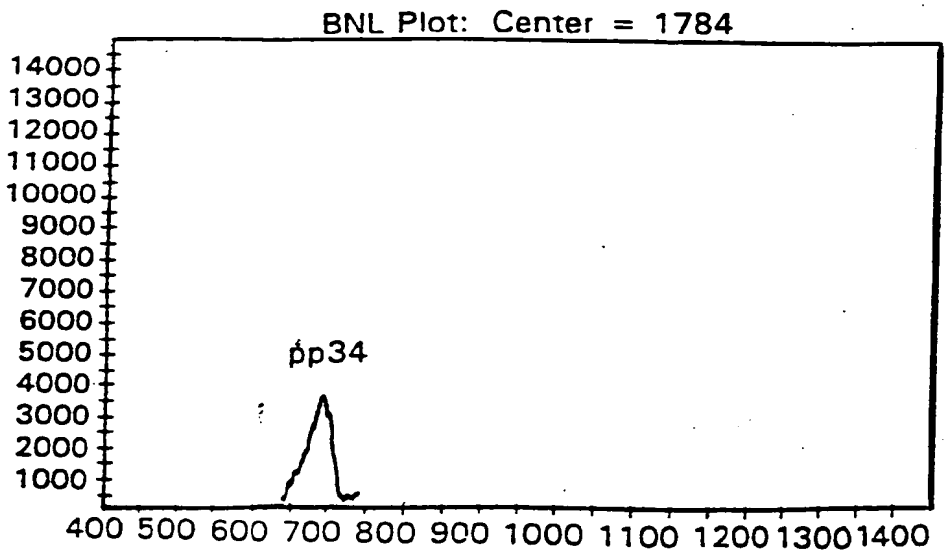


FIG. 34(a)

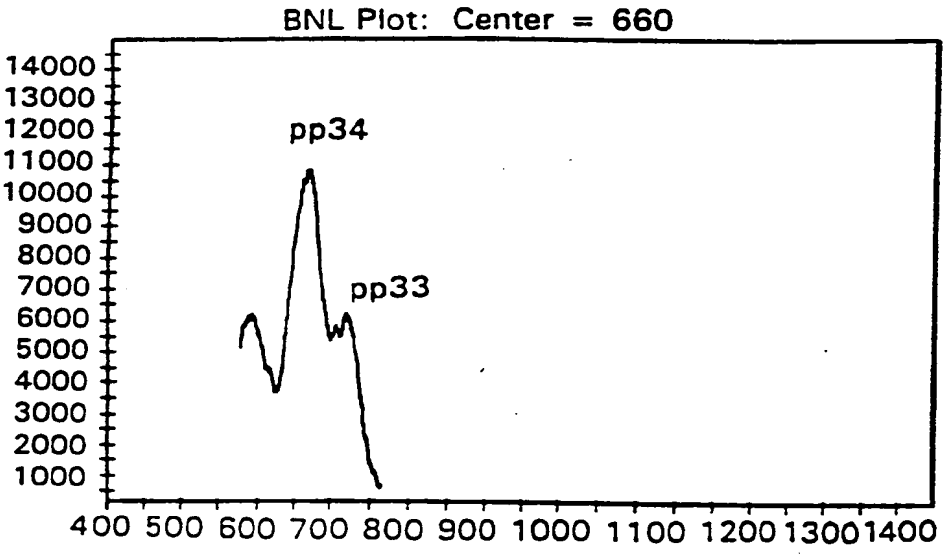


FIG. 34(c)

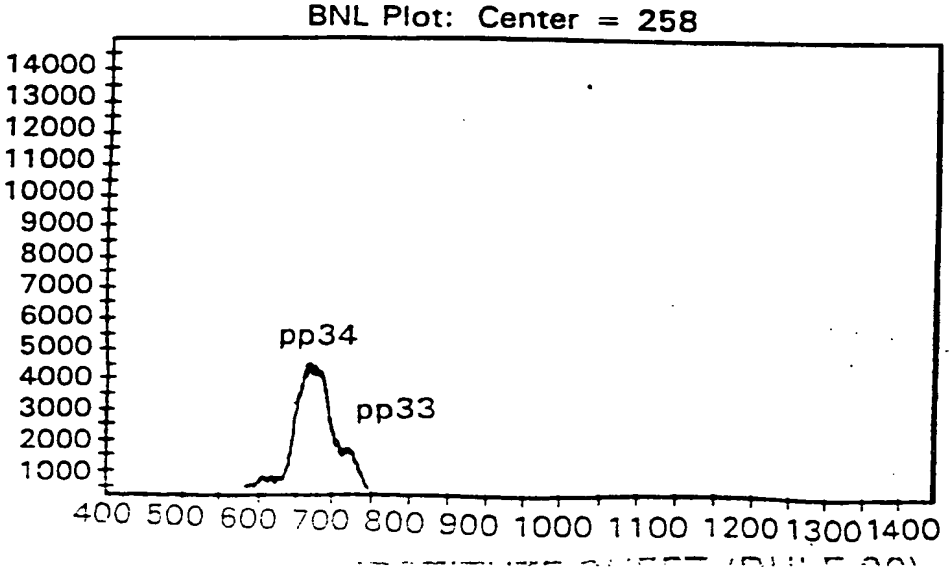


FIG. 34(e)

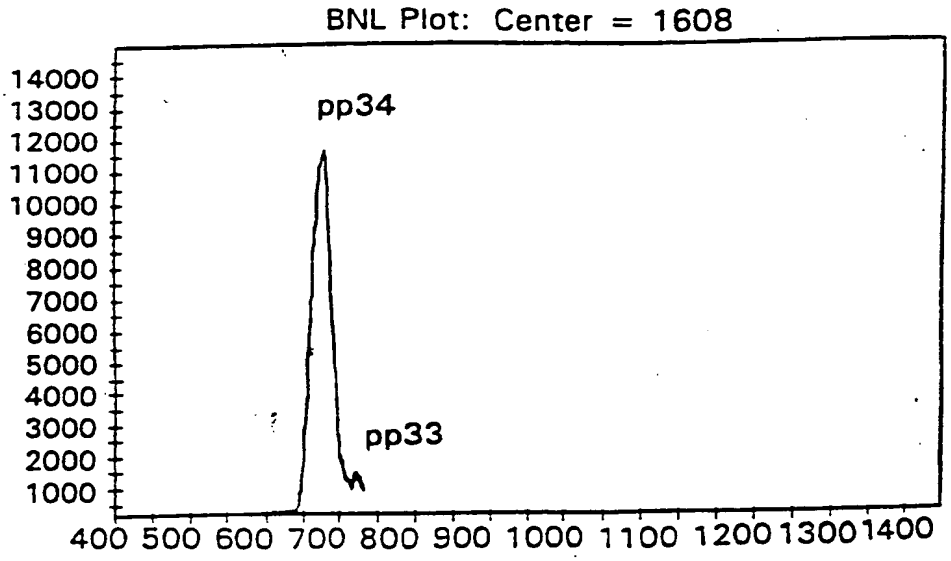


FIG. 34(b)

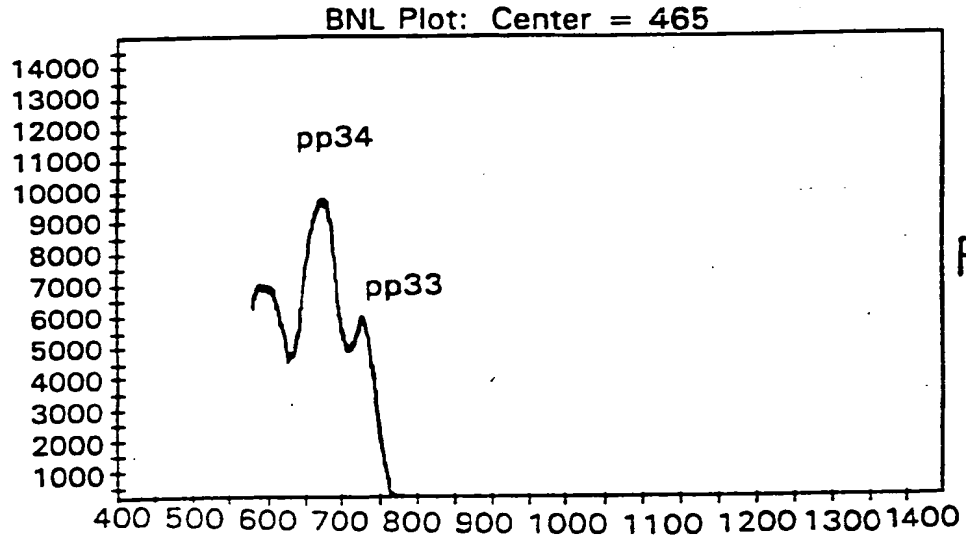


FIG. 34(d)

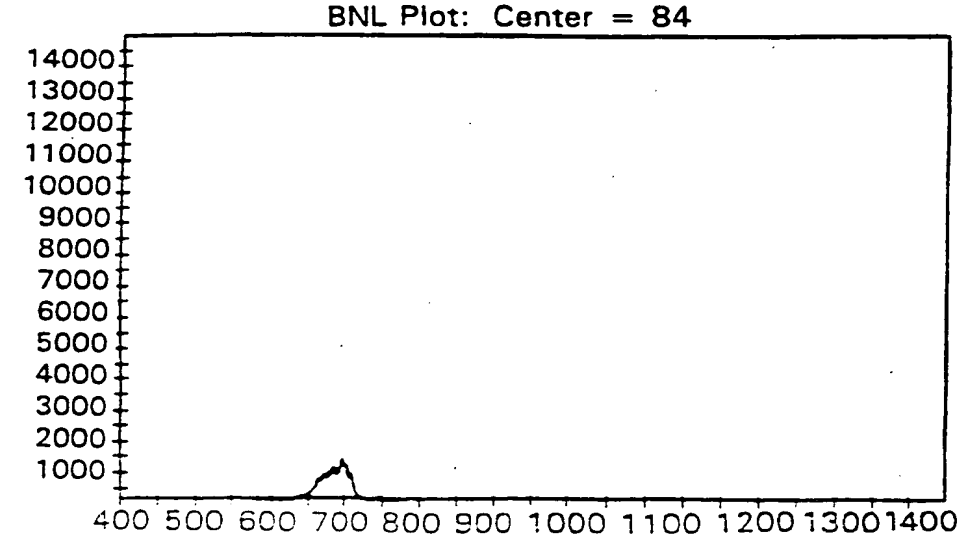


FIG. 34(f)

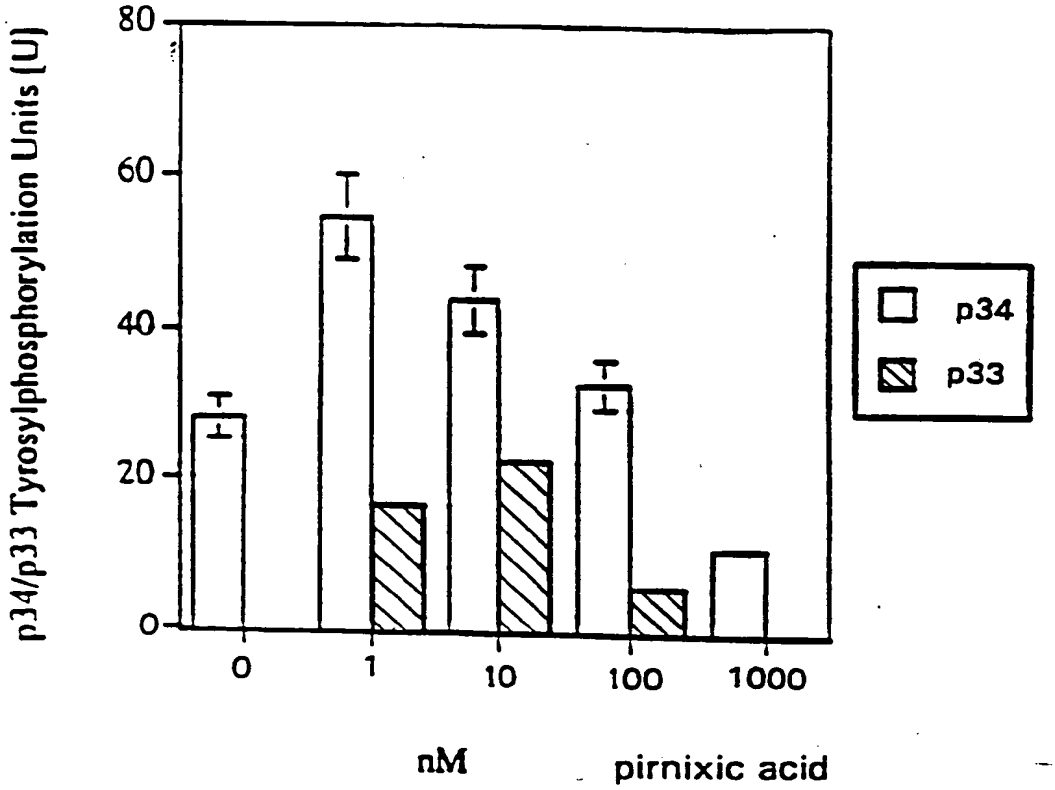

p34/p33 Tyrosylphosphorylation Units (U)
80
60
40
20
0
0
1
10
100
1000
nM
pirnixic acid
p34
p33

FIG. 35

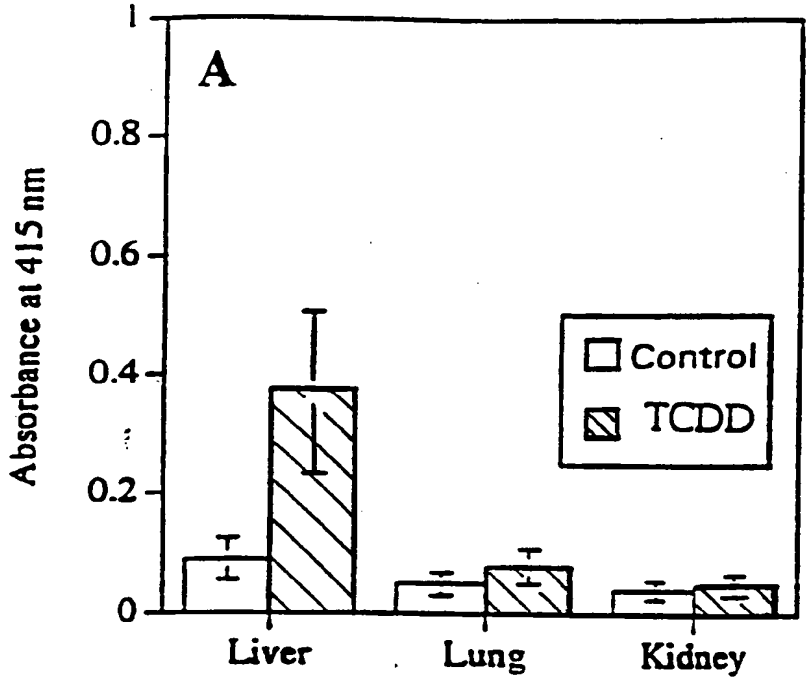


FIG. 36 (a)

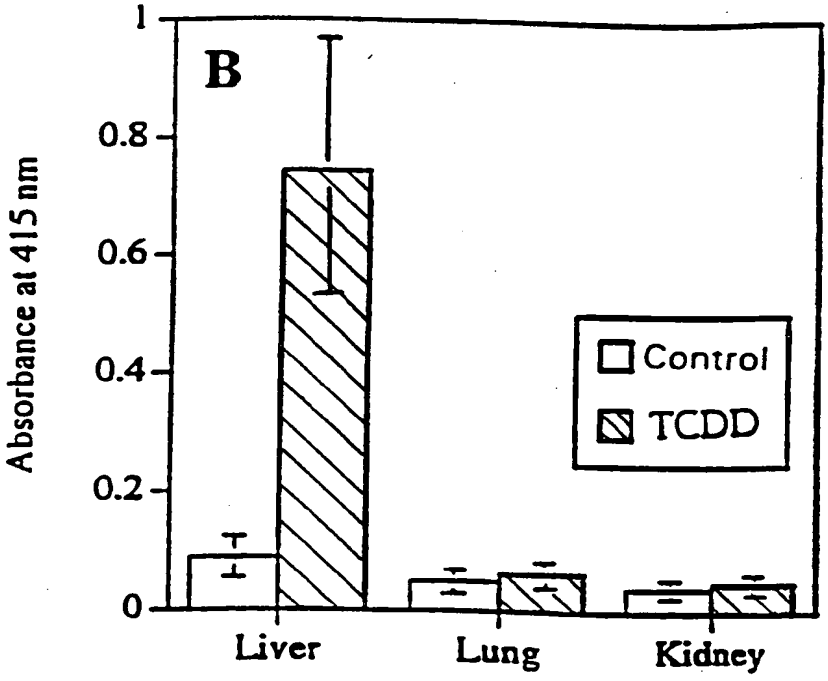


FIG. 36 (b)

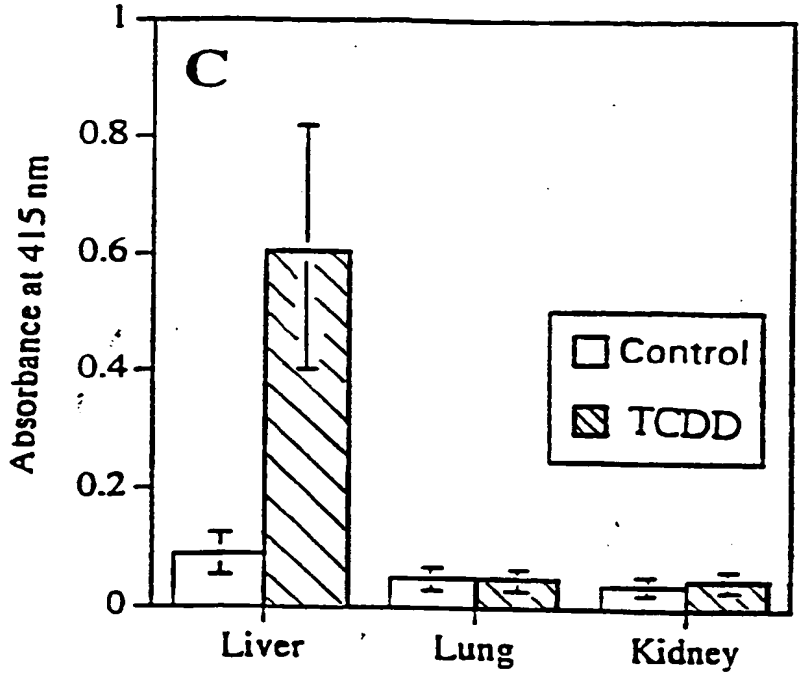


FIG. 36 (c)

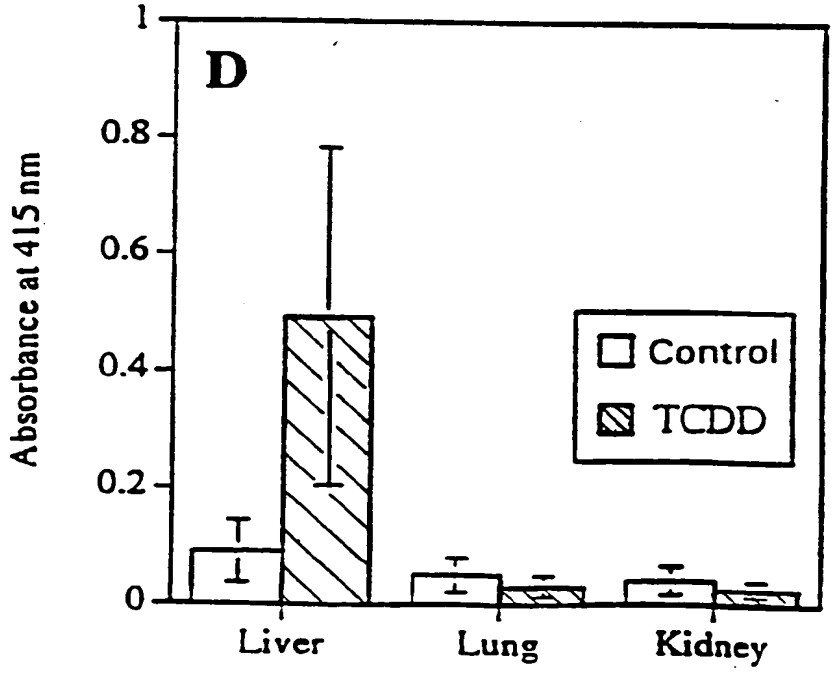


FIG. 36 (d)

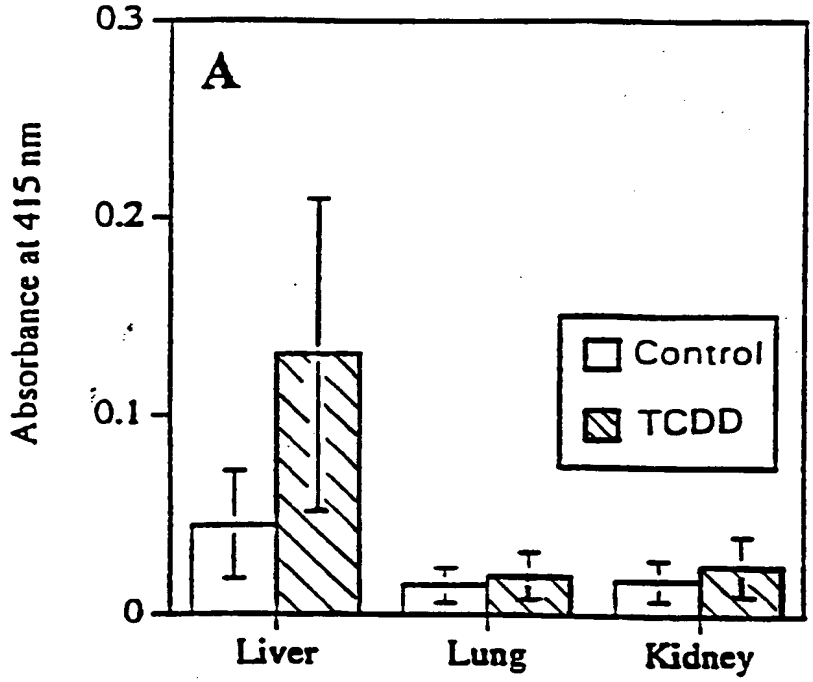


FIG. 37 (a)

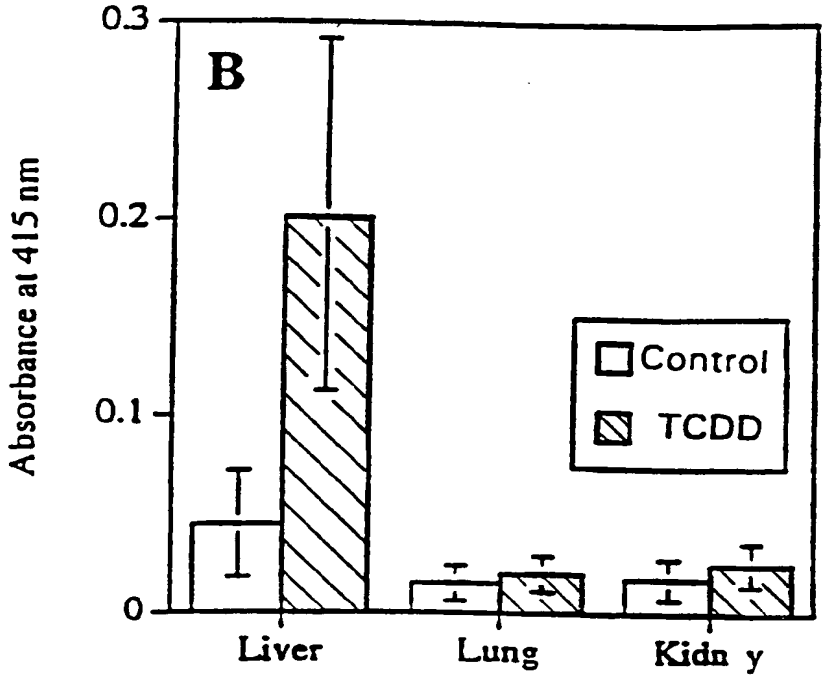


FIG. 37 (b)

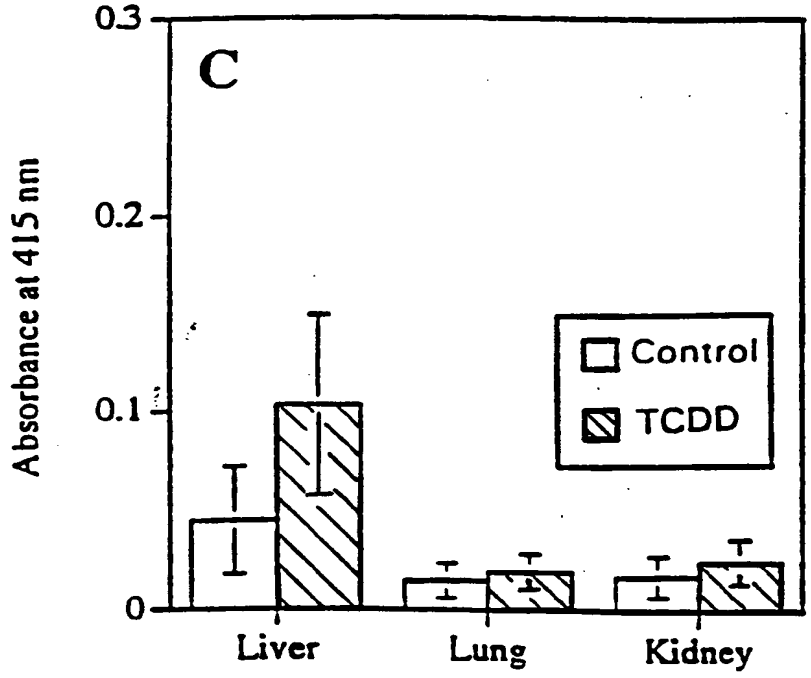


FIG. 37(c)

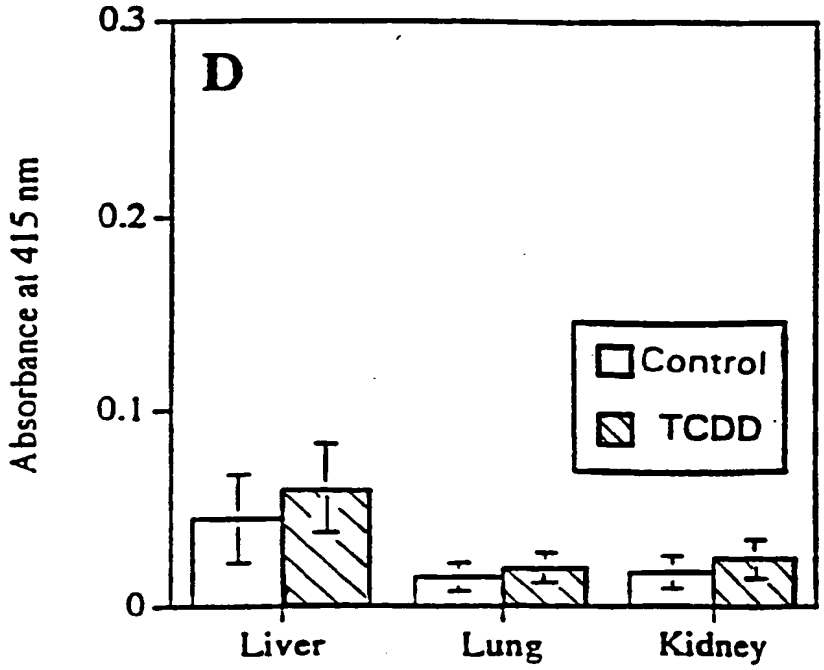


FIG. 37(d)

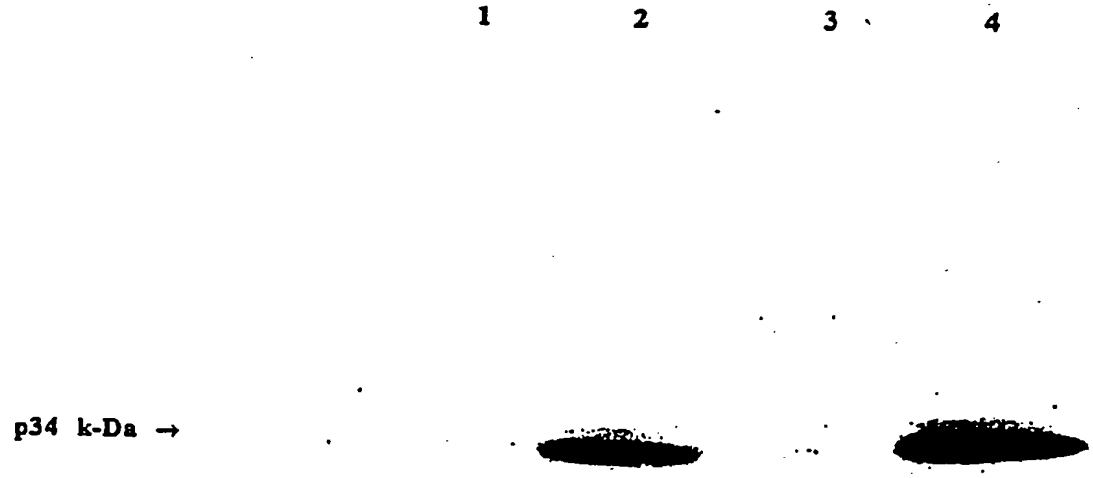


Fig 38

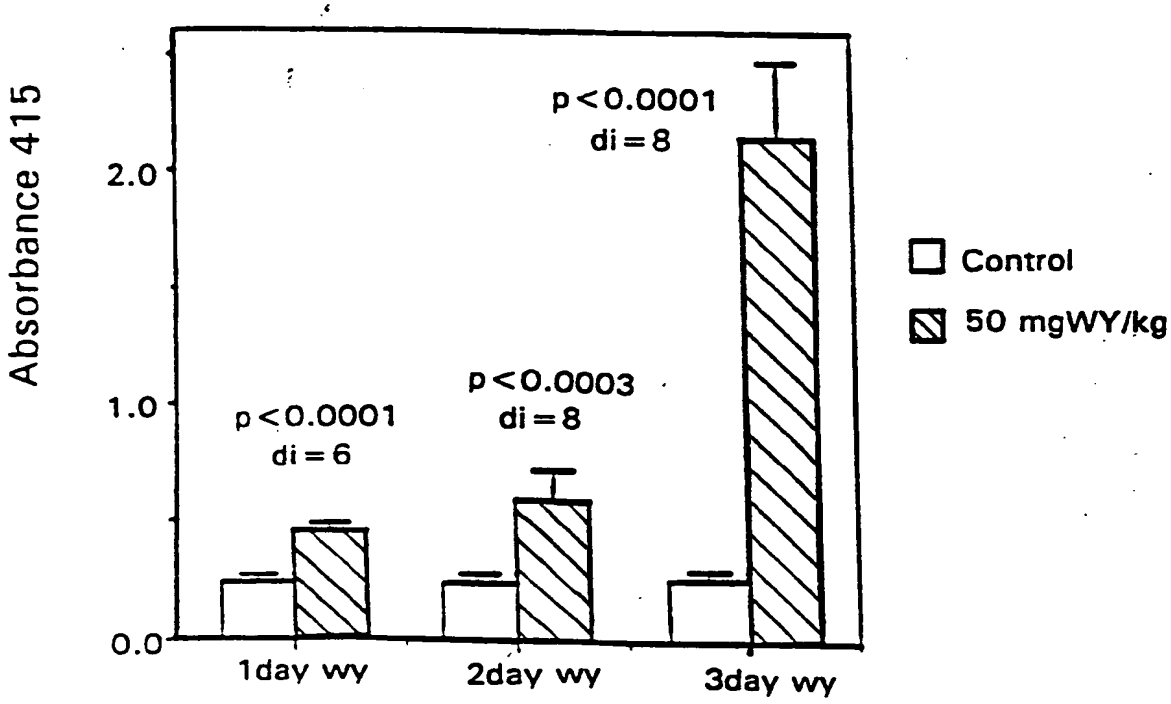
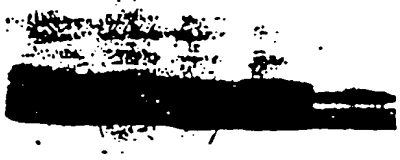

Absorbance 415
2.0
1.0
0.0
p<0.0001
di=8
p<0.0003
di=8
p<0.0001
di=6
1day wy
2day wy
3day wy
Control
50 mgWY/kg

FIG. 39



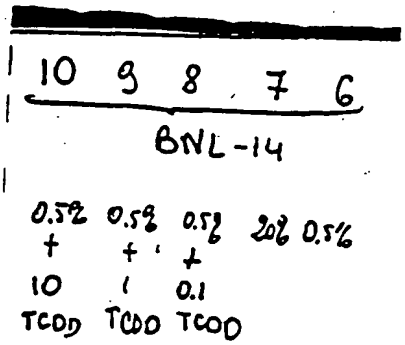


FIG 40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00961

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/574, 33/573, 33/53; C12Q 1/50
US CL :435/7.23, 7.4, 7.9, 17; 436/64, 813
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.23, 7.4, 7.9, 17; 436/64, 813

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG: Medline, Biosis, Embase, Cancerlit, Derwent; APS
search terms: cyclin, kinase, cancer, carcinoma, malig?, neoplas?, transform?, p34cdc2, dependent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	Cancer Research, Volume 53, No. 22, issued 15 November 1993, Z.A. Khatib et al, "Coamplification of the *CDK4* Gene with *MDM2* and *GLI* in Human Sarcomas", p. 5535-5541. See the abstract, the right-hand column of page 5535, the right hand column of page 5539, and the left-hand column of page 5540.	1-45
Y, P	Cancer Research, Volume 53, No. 3, issued 01 February 1993, S. Mishra et al, "*O*-Phospho-L-tyrosine Inhibits Cellular Growth by Activating Protein Tyrosine Phosphatases", pages 557-563. See the Abstract.	1-45

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 MAY 1994	23 MAY 1994

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00961

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Annu. Meet. Am. Asso. Cancer Res., Volume 33, issued 1992, M.J. Marcote et al, "Mechanisms of Activation of Cyclin-Dependent Kinases", Abstract No. A575, see the entire abstract.	1-45
Y, P	Proc. Annu. Meet. Am. Assoc. Cancer Res., Volume 34, issued 1993, M.R. Hellmich et al, "The Kinase CDK5 is Expressed in Breast, Lung and Prostate Tumor Cells in Contrast to Its Selective Expression in Postmitotic Neurons", Abstract No. A244, see the entire abstract.	1-45